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QUANTITATIVE PHARMACOPROTEOMICS  
INVESTIGATION OF ANTI-CANCER DRUGS IN  
MOUSE

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Quantitative pharmacoproteomics investigation of anti-cancer drugs in  
mouse

Development and optimisation of proteomics workflows for evaluating the  
effect of anti-cancer drugs on mouse liver

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## **Abstract**

Hamza Mohammad Ali Abumansour

Quantitative pharmacoproteomics investigation of anti-cancer drugs in mouse

Development and optimisation of proteomics workflows for evaluating the effect of anti-cancer drugs on mouse liver

Keywords: pharmacoproteomics, anti-cancer drugs, shotgun, cytochrome P450, mass spectrometry, drug toxicity, mouse liver, drug-induced liver injury, pheromone

Minimizing anti-cancer drug toxicity is a major challenge for the pharmaceutical industry. Toxicity is most frequently due to either the direct interaction of the drug on previously unidentified targets or its conversion to metabolites by drug metabolizing enzymes (e.g. CYP450 enzymes) that cause cellular, tissue or organ damage. Pharmacoproteomics is beginning to take a central role in studying changes in protein expression corresponding to drug administration, the results of which, inform about the mode of action, toxicity, and resistance in pre-clinical and clinical stages of drug development. The main aim of this research is to apply comparative proteomics studies on livers from male and female mice xenograft models treated with major anti-cancer drugs (5-flourouracil, paclitaxel, cisplatin, and doxorubicin) and CYP inducer, TCPOBOP, to investigate their effect on protein expression profiles (proteome). Within this thesis, an attention is paid to optimise a highly validated proteomics workflow for biomarker identification.

Proteins were extracted from liver microsomes of mice treated in two separate sets; Set A – male (5-fluoruracil, doxorubicin, cisplatin and untreated) or Set B – female (5-fluoruracil, paclitaxel, TCPOBOP and untreated) using cryo-pulverization and sonication method. The extracts were digested with trypsin

and the resulting peptides labelled with 4-plex iTRAQ reagents. The labelled peptides were subjected for separation in two-dimensions by iso-electric focusing (IEF) and RP-HPLC techniques before analysis by mass spectrometry and database searching for protein identification.

Set A and Set B resulted in identification and quantification of 1146 and 1743 proteins, respectively. Moreover, Set A and Set B recovered 26 and 34 cytochrome P450 isoforms, respectively. The microsomal changes after drug treatments were quite similar. However, more changes were observed in the male set. Up-regulation of MUPs showed the greatest distinction in the protein expression patterns in the treated samples comparing to the untreated controls. In Set A, 5-fluoruracil and cisplatin increased the expression of three isoforms (MUP1, 2, and 6), whereas doxorubicin has increased the expression of four isoforms (MUP1, 2, 3, and 6). On the other side, only TCPOBOP in Set B has increased the expression of two isoforms (MUP1 and 6).

Our findings showed that the expression of MUP, normally involved in binding and excretion of pheromones, have drug- and sex-specific differences. The mechanism and significance of MUP up-regulation are ambiguous. Therefore, the impact of each therapeutic agent on MUP and xenobiotic enzymes will be discussed.

## **Dedication**

**To**

**The soul of my beloved mother**

الى روح

أُمِّي الغالية

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## List of abbreviations

2D-DIGE	Two-dimensional differential gel electrophoresis
3MC	3-methylcholantrene
5FU	5-fluorouracil
AACS	Acetoacetyl-CoA synthetase
ACDs	Anti-cancer drugs
AhR	Aryl hydrocarbon receptor
ARG	Arginase
ASP	Aspartyl aminopeptidase
ASS	Argininosuccinate synthase
ATP	Adenosine triphosphate
AX	Anion exchange
BCKD	Branched-chain alpha-keto acid dehydrogenase complex
BOMCC	7-benzyloxy-methyloxy-3-cyanocoumarin
BSA	Bovine serum albumin
Ca <sup>2+</sup>	Calcium ion
CA	Carbonic anhydrase
CAPs	Cytoplasmic associated proteins
CAR	Constitutive androstane receptor
CASH	Chemotherapy induce steatohepatitis
CAT	Catalase
CE	Capillary electrophoresis
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CHCA	$\alpha$ -cyano-4-hydroxycinnamic acid
CI	Chemical ionization
CID	Collisionally induced dissociation
CILI	Chemotherapy-induced liver injury
CIS	Cisplatin
COMT	Catechol-O-methyl transferase
CP	Cryo-pulverization
CP+S	Cryo-pulverization and sonication
CPS	Carbamoyl-phosphate synthase
CPT	Carnitine O-palmitoyltransferase
CrEL	Cremaphor-EL
CX	Cation exchange
CYP450	Cytochrome P450
D	Dounce homogenizer
DILI	drug induced liver injury
DLD-1	Human colon adenocarcinoma
DMEs	Drug metabolizing enzymes
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence

EDTA	Ethylenediamine-tetraacetic acid
EMOCC	7-ethoxymethoxy-3-cyanocoumarin
ER	Endoplasmic-reticulum
ESI	Electrospray ionization
FAB	Fast atom bombardment
FABL	Fluoro-beta-alanine
FABP	Fatty acid-binding protein
FAS	Fatty acid synthase
FASP	Filter-aided sample preparation
Fe <sup>2+</sup>	Ferrous ion
Fe <sup>3+</sup>	Ferric ion
FMO	Flavin-containing monooxygenases
FT-ICR	Fourier transform-ion cyclotron resonance
G6PD	Glucose-6-phosphate dehydrogenase
GH	Growth hormone
GO	Gene ontology
GRAVY	Grand average of hydropathy
GSTs	Glutathione S-transferases
H	Disperse homogenizer
HER-2	Human epidermal growth factor receptor-2
HNF-4 $\alpha$	Hepatocyte nuclear factor-4 $\alpha$
HPLC	High-performance liquid chromatography
IAA	Iodoacetamide
IEC	Ion exchange
IEF	Isoelectric-focusing
IL-6	Interleukin 6
IMPs	Integral membrane proteins
iNOS	Inducible nitric oxide synthase
IPG	Immobilized pH gradient
IPI	International Protein Index
IT	Ion trap
iTRAQ	Isobaric tag for relative and absolute quantitation
LC	Liquid chromatography
LDH	Lactate dehydrogenase
LIT	Linear ion trap
MALDI	Matrix assisted laser desorption/ionization
MAO	Monoamine oxidase
MOWSE	Molecular weight search engine
MRM	Multiple reaction monitoring
MS	Mass spectrometer
MudPit	Multi-dimensional protein identification technology
MUP	Major urinary protein
NaN <sub>3</sub>	Sodium Azide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NATs	N-acetyltransferases

NO	Nitric oxide
NQO	NADPH-quinone oxidoreductase
OG	OFF-GEL
PAGE	Polyacrylamide gel electrophoresis
PANC-1	Human pancreatic carcinoma
PAP	Prostatic acid phosphate
PAX	Paclitaxel
PB	Phenobarbital
PBS	Phosphate buffered saline
PCIS	Precursor ion selector
PD	Plasma desorption
PDIs	Protein disulfide isomerases
pI	Isoelectric point
PIC	Protease inhibitor cocktail
PMF	Peptide mass fingerprinting
PSMs	peptide spectrum matches
PTMs	Post-translational modifications
PXR	Pregnane X receptor
RFU	Relative fluorescent units
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAX	Strong anionic exchange
SCX	Strong cation exchange
SDS	Sodium dodecyl sulphate
SILAC	Stable isotope labelling by amino acids
SLC	Solute carrier transporters
SOD	Superoxide dismutase
SULTs	Sulfotransferases
TBP	Tributylphosphine
TCA	Tricarboxylic acid cycle
TCEP	Tris-(2-carboxyethyl)-phosphine
TCPOBOP	1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene
TEAB	Triethylammonium bicarbonate
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TMPT	Thiopurine S-methyl transferase
TNF $\alpha$	Tumour necrosis factor
TOF	Time of flight
Tris	Tris-hydroxymethyl aminomethane
TXNDC	Thioredoxin domain-containing protein
UGTs	UDP-glucuronosyltransferase
UPB1	Beta-ureidopropionase
URP	Unfolded response protein

## List of appendices

*A soft copy of appendices is attached in a CD along with this thesis.*

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## Chapter one: General introduction

# **1 General introduction**

## **1.1 Scope of pharmacology**

In general terms, pharmacology is a scientific field that plays a crucial role in linking various disciplines of biomedical sciences such as; biochemistry, cell biology, physiology, pathology, and genetics together in order to study the effects of the drug on living organism (Rang et al., 2014). It's a basic science for medicine as well as for pharmacy, nursing, dentistry, and veterinary medicine in the study of drugs. According to the US FDA, a drug is defined as a chemical substance which has a physiological effect intended for use in diagnosis, cure, mitigation, treatment, or prevention of disease (Li, 2015). More so, a World Health Organisation scientific group defined the drug as “any substance or product that is used or intended to be used to modify or explore the physiological systems or pathological states for the benefit of recipient”.

In spite of the various general definitions for pharmacology, pharmacological studies aim to deeply understand the mechanisms by which drugs interact with human systems to enable the rational use of pharmacological agents for treatment and diagnosis of disease (Brenner and Stevens, 2013). In other words, pharmacology looks at every aspect of the relationship of the drug with the body, starting from the time that a drug enters the body to the point of its excretion. This relationship is covered by the means of two important and interesting areas of pharmacology; pharmacokinetics and pharmacodynamics.

Pharmacokinetics involves drug concentration changes over time in relation to the dose of the drug in one or more different parts of the body as a result of drug absorption, distribution, metabolism or excretion. On the other hand,

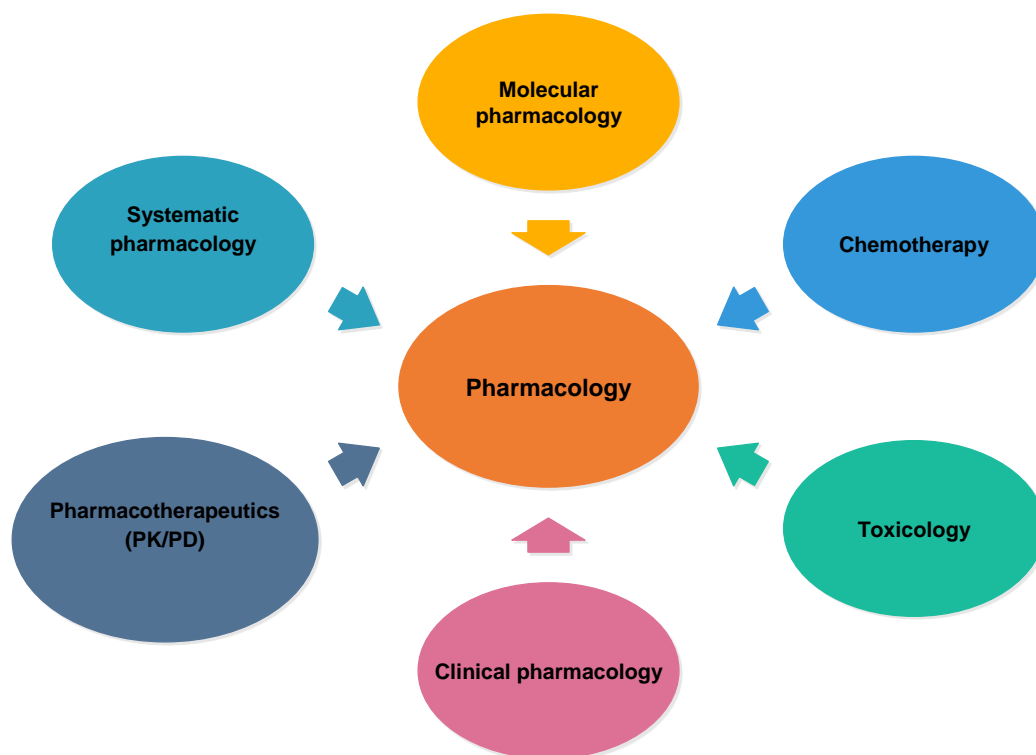


pharmacodynamics involves what the drug does to the body once it reaches its target and the consequential biological effects produced as a result of the interaction. However, as a distinctive science, pharmacology can be studied at all levels, starting from the molecules in the cell to the organ to the whole body. Genomics and proteomics are new approaches in pharmacology that can help in developing personalized medicine by exploring the character of genetic variability. Pharmacology can be further subdivided into: chemotherapy, pharmacogenomics, pharmacoeconomics, neuropharmacology, pharmacoepidemiology, and clinical pharmacology, as well as toxicology (Figure 1.1).

A drug exerts its action once binds to its target site, and to achieve that, typically, the drug should first enter the body and distribute via the bloodstream to reach the target site. After that, the drug is eventually eliminated from the body after being modified/degraded by a set of specific metabolizing enzymes, where the metabolism process refers to “a chemical or structural biotransformation of endogenous and/or xenobiotics compounds like drugs”. Indeed, defined metabolizing enzymes are responsible for changing drugs into more water-soluble metabolites, thereby facilitating their excretion through urine or bile (Ioannides, 2008). However, some drugs are metabolised before they exert their action, others are metabolised subsequently after exerting their action, and still other drugs are not subjected to any of the metabolising processes.

For those being metabolised, the resultant products can be either; inactive or more active than the parent drug in therapeutic activity or toxic. However, some drugs are designed to be inactive when being administrated to the

human body, where they are metabolised into active metabolites producing the desired therapeutic effect and are called prodrugs.



**Figure 1-1. Major branches of pharmacology. Pharmacotherapeutics concerns studying drug's pharmacodynamics (systemic and cellular effects) and pharmacokinetic (absorption, distribution, metabolism, and excretion) properties. Systematic pharmacology such as; neuro-, cardio-, immuno- and other pharmacology. Clinical pharmacology concerns the aspects of drug efficacy and safety.**

The liver is the primary organ in the body responsible for the metabolism of endogenous and exogenous compounds via a large group of hepatic enzymes, collectively referred to as drug metabolizing enzymes (DMEs). DMEs are involved in the process of modifying a wide variety of exogenous (e.g. pharmaceutical agents, chemical carcinogens, lipophilic xenobiotics) and endogenous (e.g. steroids, fatty acids, prostaglandins, vitamin D3) molecules that are biologically active (Pelkonen et al., 2008). Generally, drug biotransformation reactions can be divided into two main phases (Phase I and

Phase II) and each phase is accomplished by a unique set of metabolic enzymes.

Cytochrome P450 family (CYP450) is the most significant family that belongs to Phase I family and is responsible for metabolizing more than 60% of clinically used drugs, resulting in polar metabolites that can be excreted in urine or undergo further conjugation reactions in Phase II (Kenaan et al., 2010). However, some drugs might undergo either phase I only or just phase II metabolism pathway, but ordinarily, the drug undergoes phase I and then phase II sequentially.

The level of CYP450 enzymes controls the rate at which many drugs are metabolised. They have limited capacity to metabolise drugs, so they can become overloaded when a high dose of the drug is given. Furthermore, many substances (e.g. drugs and foods) may affect the CYP450 enzymes expression level or activity. If these substances reduce the ability of these enzymes to metabolise a drug, then that drug's effects (including side effects) will increase. On the other hand, if the substances induce the ability of the enzymes to metabolise a drug, the drug's effects will decrease (Lim et al., 2013).

In conclusion, drug metabolizing enzymes activity may result in:

- (i) Bioactivation of inactive drug into reactive metabolites (positive activation, conversion of prodrug to drug).
- (ii) Potentiating the activity of drug by giving a highly reactive intermediate (metabolic activation).

- (iii) Conversion of an active drug into equally active metabolites (no change activation).
- (iv) Inactivation of active drugs into inactive metabolite (negative activation).

Examples of these biological conversions of Phases I and II are shown in Table 1.1.

In the pharmaceutical “pipeline” of new drug development, studying DMEs is a cornerstone in the evaluation of drug efficacy and safety in experimental animals and humans. DMEs are important biomarkers in the occurrence of a number of drug interactions that may result in drug toxicities, pharmacological effect failure, and adverse drug reactions. Identifying whether a drug has the potential for inhibiting or inducing other DMEs, can minimise or even prevent clinically significant interactions from occurring (Cascorbi, 2012).

**Table 1-1. Examples of the different mechanism of Phases I and II metabolizing processes.**

<b>Biological process</b>	<b>Example</b>
<b>Positive activation</b>	Conversion of cyclophosphamide to the pharmacologically active form (4-hydroxycyclophosphamide).
<b>Metabolic activation</b>	Production of N-acetyl-benzoquinonimine, the product of paracetamol metabolism.
<b>No change-activation</b>	Conversion of digitoxin to digoxin.
<b>Negative activation</b>	Inactivation of phenytoin to p-hydroxy-phenytoin.

Sex-related differences are another potential issue, which highlights the importance of studying DMEs, in particular, the CYP450 family. Recent studies showed that the number of pharmacological responses that are sex-specific is

more common than previously thought, which includes pharmacokinetics and pharmacodynamics, with more emphasis on the former. These differences led to individual variations in the toxicity and efficacy of the drug. However, the main consequence of sex-dependant pharmacokinetics is the sex-based variations in drug metabolism, which may be due to their hormonal effects on physiological processes. Concerning the differences in pharmacokinetics, Phase I and II subfamily enzymes exhibited marked sex variations in *in vitro* and animal studies. Moreover, the sex variation is not only at the enzyme expression level but also at the activity level of metabolizing enzymes (Chang et al., 2011). For example, CYP3A4 which is responsible for the metabolism of about 50% of clinically used drugs from almost all therapeutic categories including anti-cancer drugs showed significantly higher activity and expression level in female than the male of human liver (Lamba et al., 2010).

The mechanistic basis for these differences has not been clarified yet, recent studies indicated that this may be due to the greater level of hormone-dependent activation and nuclear translocation of hepatocyte nuclear factor-4 $\alpha$  (HNF-4 $\alpha$ ) and pregnane X receptor in female hepatocytes compared to male hepatocytes (Zanger and Schwab, 2013, Thangavel et al., 2011). On the other hand, there are some other factors that can lead to metabolizing enzymes variation, which may include but not limited to, exposure to xenobiotics, regulation by cytokines, hormones, during disease states, and age (Zanger and Schwab, 2013).

Drugs have been investigated intensely for their effects on CYP450 enzymes expression and activity. It has been found that drugs, when administered internally, can modulate the expression of CYP450 enzymes and

subsequently, the activity of these enzymes through either inhibiting or inducing them. Enzyme inhibition signifies a decrease in the enzyme activity as result of the direct binding between drug and particular CYP enzyme either; reversibly (non-mechanism-based binding) or irreversibly (mechanism-based binding) (Pelkonen et al., 2008). Generally, the reversible inhibition can be competitive, non-competitive or uncompetitive. However, binding of the drug to a particular CYP450 enzyme to inhibit its activity is usually an artefact and occurs competitively (Ring et al., 2014). On the other hand, induction is to increase the amount of specific CYP450 enzymes (synthesis) and subsequently speed up the rate of metabolic elimination of drug itself or other concomitant drugs (Martikainen, 2012).

Studying the changes in the expression and activity of membranous proteins such as CYP450 enzymes as a result of drug administration or disease status remains the main challenge. The low abundance of the CYP450 enzymes, their location, complex family profile, structure and physiochemical properties are all factors that provoke such challenges in this area of research (Donoghue et al., 2008, Golizeh and Sleno, 2013).

Recently developed proteomics approaches have significantly increased the efficiency and applicability of mapping drug-protein interactions. The scope of proteomics is not limited to a list of proteins, but rather profiles every protein expressed in a target cell or tissue at any time in response to any stimulus such as exposure to a drug. Therefore, proteomics has been used in both, *in vivo* and *in vitro* studies for drug efficacy and safety (Barbosa et al., 2012).

Pharmacoproteomics is a branch that deals with the application of proteomics in the field of drug discovery and development, in addition to the assessment

of drug administration (D'Alessandro and Zolla, 2010). It is known as a branch of proteomics, rather than a branch of pharmacology. It is used to understand the efficacious effects as well as side effects of drugs on organ proteome (D'Alessandro and Zolla, 2010). However, the proteome is defined as the complete set of proteins produced by a particular cell, tissue, or organ that might be affected as a result of drugs administration, pathophysiological condition or other (Bateson et al., 2011). Pharmacoproteomics aims to accelerate the drug development process, reduce costs, and provide tools for better management of diseases through understanding the effects of established drugs on protein expression in biological systems. In order to achieve that, proteomics in drug studies must be able to: (i) verify new drug targets, (ii) reveal the exact molecular mechanism of drug action including activity and toxicity, (iii) develop protein biomarkers and assays for assessment of drug efficacy, and (iv) identify consequences of toxicity for both preclinical and clinical applications (Lee et al., 2011).

The liver proteome has been analysed in various comparative pharmacoproteomics studies to investigate whether drugs are able to change protein expression, particularly xenobiotic enzymes, and subsequently cause liver toxicity. Since 70% of blood supply reaches it by the portal vein, carrying the ingested xenobiotic from stomach and intestine, liver is a potential target organ for drug and toxin accumulation (Coleman M.D, 2010). Drug toxicity in the liver can be emphasized by drug-induced liver injury (DILI). Indeed, DILI is a serious matter for new drug candidates as well as for already marketed drugs.

## **1.2 Cancer**

The term cancer refers to a unique set of diseases in which cells divide uncontrollably by disregarding the normal rules of cells division, leading to the invasion of surrounding tissues and metastasizing to distant sites of the body, causing the death of the host at the end stage (Weinberg, 2013). Normally, cells grow and divide in a controlled and orderly way to produce more cells when the body needs them. However, sometimes cells overcome the rules of the division to become abnormal and keep dividing to form more cells without control or order, creating a mass of excess tissue called a tumour. Tumours can be benign or malignant. Contrary to the benign tumour, malignant can spread to surrounding cells and other parts of the body (metastasis) via the bloodstream or lymphatic system (Ruddon, 2007).

Cancer treatment can take numerous approaches including surgery, radiotherapy, photodynamic therapy, chemotherapy, immunotherapy, and hormone therapy or a combination (e.g. radiosurgery). Cancer treatment strategy depends mainly on the nature of cancer and how far it has progressed (Makropoulou, 2016).

Chemotherapy is a subdivision of pharmacology, which deals with the effects of drugs used to kill neoplastic cells. In oncology, chemotherapy is a preferred option due to the direct capability of targeting tumour cell death by necrosis or apoptosis, vascular damage leading to tissue ischemia and resultant target cell death, or immune modulation, or a combination of these (Ricci and Zong, 2006). Anti-cancer drugs (ACDs) comprise a number of chemical agents that are used to halt the progression of cancer by interfering with either cell division



or DNA synthesis, usually causing severe side effects (Akhdar et al., 2012). Based on the mechanism of action, the traditional anti-cancer agents have been categorized into metalating-intercalating agents (cisplatin, cyclophosphamide), antimetabolite (5-fluorouracil, mercaptopurine), topoisomerase I inhibitors (topotecan), topoisomerase II inhibitors (anthracyclines; doxorubicin), alkaloid (taxol, vincristine), and others (Akhdar et al., 2012).

Contrary to traditional therapies, modern anti-cancer agents have distinctive mechanisms of action against one or more pivotal cellular pathways involved in the cancer process (Giamas et al., 2010a). Therefore, their selectivity and efficacy are enhanced, where toxicity has reduced amongst others. The current targeted therapies include; immunotherapy, tyrosine kinase inhibitors, hormone therapy, etc. (Widmer et al., 2014).

Immunotherapy or monoclonal antibody targets a specific host antigen such as prostatic acid phosphate (PAP) in prostate cancer by Sipuleucel-T and human epidermal growth factor receptor-2 (HER2) in breast cancer by trastuzumab (Kantoff et al., 2010, Hudis, 2007). Imatinib is a tyrosine kinase inhibitor which blocks the oncogenic signalling cascade causing the cells to die (Buchdunger et al., 2002). Other drugs are still in early stages of drug development such as drugs affecting histone-modifying enzymes (Giamas et al., 2010b, Razak et al., 2011).

The effects of chemotherapies have been investigated intensively in preclinical and clinical phases of drug development on liver models for any possibility of drug-protein or drug-drug interactions. Such investigation has been of high

interest owing to the aggressive natures of both cancer disease and anti-cancer drugs. Furthermore, since cancer is a heterogeneous and complex disease, it is typically managed with a combination of chemotherapies to overcome the problem of resistance. This results in increasing the chance of drug-drug interaction and subsequently liver injury. Besides, a number of important chemotherapeutic drugs such as cyclophosphamide, ifosfamide, tamoxifen or procarbazine are administered as prodrugs and have to be activated by defined CYP450 isoform (Preissner et al., 2012).

## **1.3 Liver toxicity**

### **1.3.1 Liver and drug metabolizing enzymes**

The liver is one of the largest organs in the body, it plays an important role in different biological processes such as homeostasis in the body, production of various plasma proteins, balancing blood sugar and pH, etc. (He, 2005). In pharmacology, the role of the liver is highly significant. It is the main organ in the body responsible for metabolizing various endogenous and exogenous compounds, even though other organs can participate in the metabolism process, such as; lungs, skin, kidney, and the gastrointestinal tract. The liver receives about 70% of the blood supply by the portal vein that carries the ingested xenobiotics, such as anti-cancer agents, from the stomach and intestine. As a result, liver is a potential organ for xenobiotic and toxin accumulation (Pelkonen et al., 2008).

The majority of drugs are metabolised in the liver, particularly in the smooth endoplasmic reticulum (ER) of the hepatocytes. Once a drug is absorbed from

the GI tract, it may undergo a first-pass or systemic metabolism, where the liver or gut wall metabolises a large portion of the drug before it circulates in the blood. The first-pass metabolism can dramatically reduce the drug's bioavailability (Gibson and Skett, 2001).

Drug metabolism process involves a chemical or structural modification of drug by DMEs in order to change it into more water-soluble metabolites (Pelkonen et al., 2008). Generally, there are two metabolic pathways for drugs transformation in the liver: Phase I (oxidation, reduction, and hydrolysis) and Phase II (conjugation) (Pelkonen et al., 2008).

Phase I reactions, which are preferably described as “functionalization reactions”, take place through either; introducing a new polar functional group like hydroxyl (-OH), amine (-NH<sub>2</sub>) or carboxylic (-COOH) to the parent drug (oxidation); or modifying an existing functional group in order to be more polar (reduction); or unmask existing polar functional group (hydrolysis). Resulted product is either inactive metabolite that can be easily excreted in urine or highly reactive intermediate metabolite that is conjugated with the endogenous compound in order to become water soluble (Phase II) (Guengerich and Shimada, 1991). Phase I includes different families of enzymes; cytochrome P450 (CYP450), flavin-containing monooxygenases (FMO), aldehyde dehydrogenases (ALD), monoamine oxidase (MAO), alcohol dehydrogenases, NADPH-quinone oxidoreductase (NQO) for quinones reduction and other (Puccinelli et al., 2011).

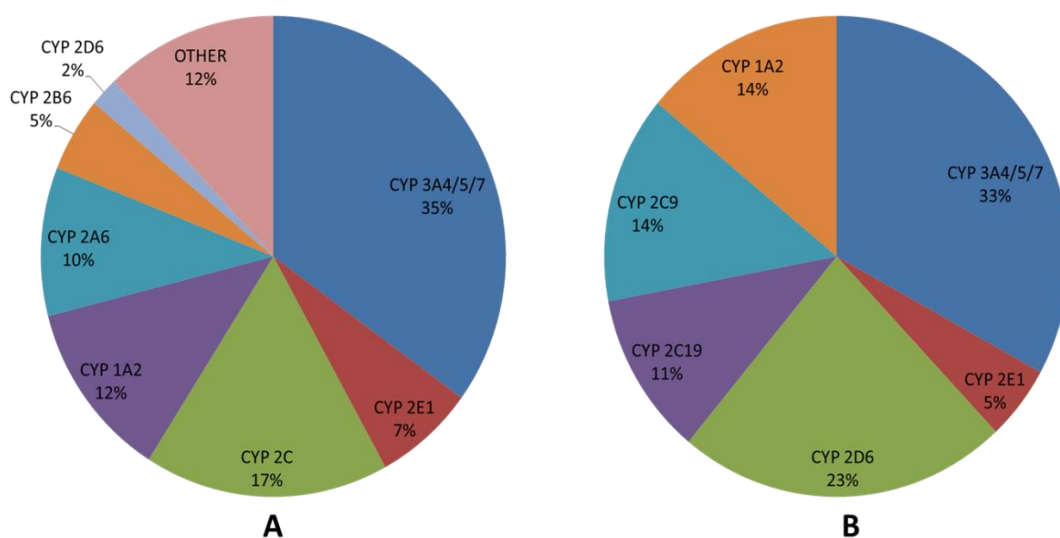
Phase II metabolizing biotransformation enzymes conjugate highly polar endogenous substrate such as; glucuronic acid, sulphuric acid, acetic acid, or

glutathione to Phase I products (reactive intermediates), making them soluble in water, and thus, easily excreted in urine and/or bile. Sulphation (sulphuric acid), glucuronidation (glucuronic acid) and glutathione conjugation are the most common classes of phase II metabolism that may occur directly on the parent compounds converting them into more hydrophilic conjugates (Jancova et al., 2010). Phase II drug metabolizing enzymes include: UDP-glucuronosyltransferases (UGTs), sulfotransferases (SULTs), N-acetyltransferases (NATs), glutathione S-transferases (GSTs) and various methyltransferases (mainly; thiopurine S-methyl transferase (TPMT) and catechol-O-methyl transferase (COMT)) (Jancova et al., 2010).

#### **1.3.1.1 Cytochrome P450 enzymes**

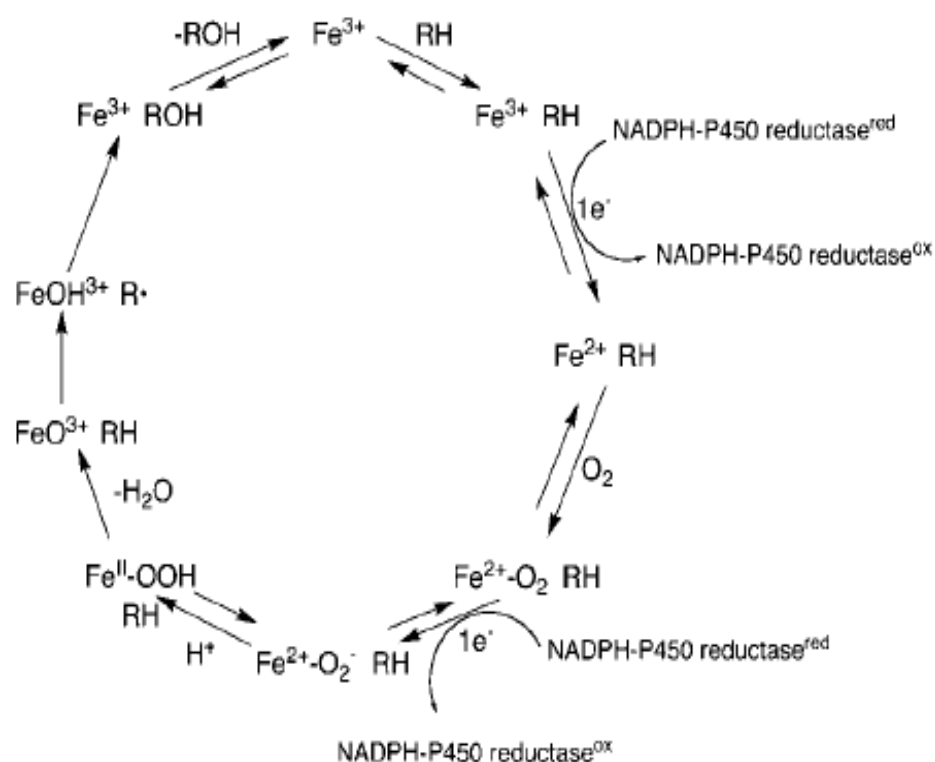
Cytochrome P450 enzymes (CYP450's) are examples of mixed-function oxidases of Phase I metabolizing enzymes, which have the ability to convert most of the drugs and other lipophilic xenobiotics to an inactive form (Ioannides, 2008). The functions of these enzymes have been found to be associated with initiation or prevention of carcinogenesis. Some CYPs are highly polymorphic enzymes that are regulated at various molecular levels. (Tamási et al., 2011). The human genome has a total of 115 CYP450 genes, among which 57 are putatively active genes and 58 are pseudogenes (Nelson et al., 2004). Although CYP450 enzymes in human are found in various tissues such as intestine, kidney, heart, lung, brain, adrenal glands, gonads, nasal and tracheal mucosa, the highest abundance and the largest number of individual CYP450 isoforms are predominantly located in the liver microsomes (Figure 1.2) (Pelkonen et al., 2008, Ghosh et al., 2010).

CYP450 enzymes most commonly undertake oxidation reactions and, to a lesser extent; reduction, hydrolysis, hydration and isomerisation reactions (Johansson, 2011). Based on the similarity of amino acid sequence, human CYP450 enzymes are classified into 18 families with at least 40% sequence homology indicated by Arabic numerals (e.g. CYP3), and 44 subfamilies with at least 55% amino acid homology indicated by a letter (e.g. CYP3A) followed by an Arabic number to identify them individually (e.g. CYP3A4) (Wang et al., 2008). CYP450s nomenclature and classification have been well described by David Nelson [<http://drnelson.utmem.edu/CytochromeP450.html>]. The CYP450 enzyme families 1–3 (2C9, 2C19, 2D6, and 3A4) are responsible for about 80% of all Phase I-dependent drug metabolisms as shown in Figure 1.2 (Pelkonen et al., 2008).



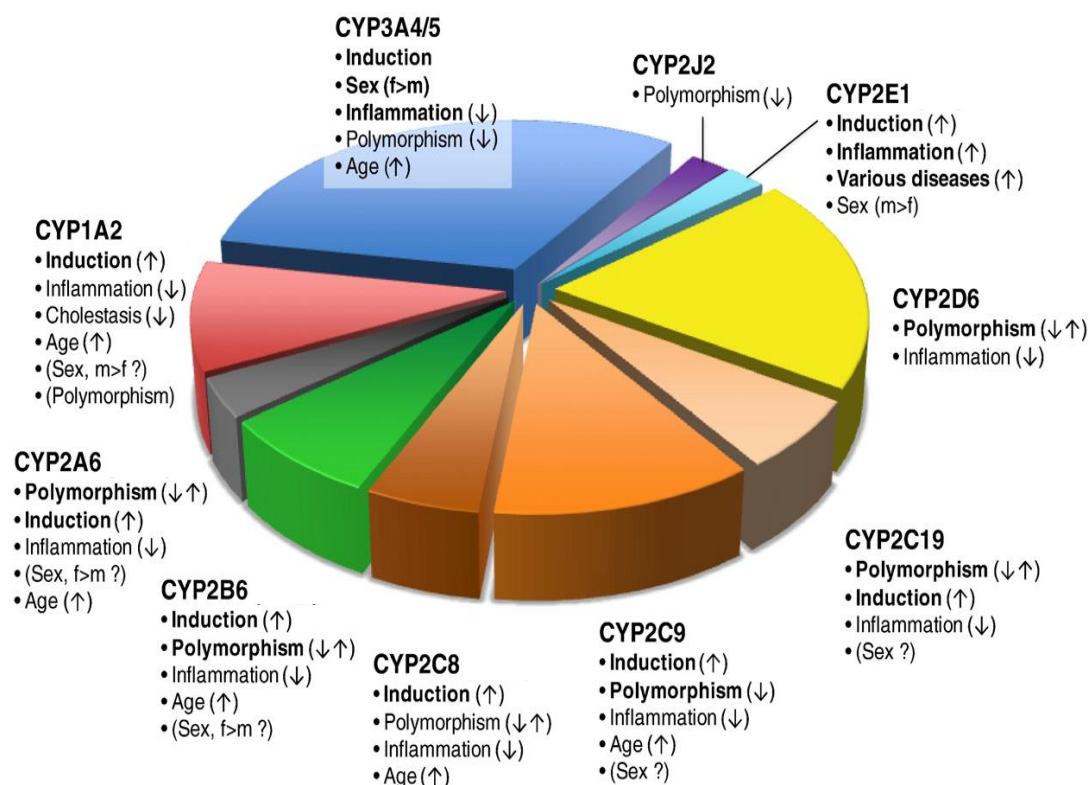
**Figure 1-2. The relative abundance of CYP450 enzymes in the liver (pie chart A). Around 50 of functionally active CYP enzymes are classified into 18 families, among these enzyme isoforms, CYP3A showed the highest abundance. However, CYP 3A4/5/7 and CYP 2D6 are responsible for metabolism the largest number of clinically used drugs (pie chart B). Modified from (Pelkonen et al., 2008, Zanger and Schwab, 2013).**

CYP450s oxidation reactions are NADPH-dependent, which enables their mono-oxygenase reaction to metabolise drugs (Figure 1.3), through coupling of the parent substrate (RH) with the active moiety of the CYP enzyme forming an oxidized complex ( $\text{Fe}^{3+}\text{-RH}$ ). NADPH oxidoreductase enzyme is a key cofactor that is responsible for transferring an electron and then reducing the heme of CYP450 ( $\text{Fe}^{3+}$ ) into ferrous ( $\text{Fe}^{2+}$ ). Moreover, and in the presence of  $\text{O}_2$ , NADPH- oxidoreductase enzyme is also responsible for the formation of ( $\text{Fe}^{2+}\text{-OOH-RH}$ ) complex, which subsequently reacts with another proton, emitting water and yielding a ferric oxene complex ( $(\text{FeO})^{3+}\text{-RH}$ ). However, cytochrome<sub>b5</sub> is able to donate an electron and form ( $\text{Fe}^{2+}\text{-OOH-RH}$ ) complex. Lastly, the ferric oxene extracts a hydrogen atom from RH and hence gives off the metabolite ROH, returning the cycle to its starting point (De Montellano, 2005, Sono et al., 1996).



**Figure 1-3. The cytochrome P450 catalytic cycle. In this reaction, the active moiety of CYP binds with the parent substrate (RH), which results briefly at the beginning in donating a proton from RH to the substrate and ROH donates the final hydroxylated metabolite. Inducing a polar hydroxyl group (-OH) to the molecule transforms a nonpolar substrate into a polar metabolite (De Montellano, 2005).**

The expression of CYP isoform is controlled by a unique set of mechanisms and factors (Figure 1.4), including genetic polymorphisms, epigenetic and non-genetic factors such as endogenous hormonal factors, sex, disease status, age as well as exposure to drugs and environmental chemicals (Zanger and Schwab, 2013). Awareness about the factors that influence the expression and activity of CYP450 enzyme is an important prerequisite to predict the pharmacokinetic parameters and subsequently the activity and safety profile of drugs that undergo metabolism by each particular enzyme.



**Figure 1-4. Important variability factors influence the expression of a fraction of clinically used drugs metabolizing CYP450 isoforms (indicated by bold type). Arrow direction indicate the possible direction of influence (↑, increased activity; ↓, decreased activity; ↑↓, increased and decreased activity) (Zanger and Schwab, 2013).**

Genetic polymorphism of DMEs is the main cause of inter-individual variability in drug response since it has been estimated to affect about 20-25% of therapeutic drugs used clinically (Ingelman-Sundberg et al., 2007, Eichelbaum et al., 2006). However, sex differences have recently become a significant factor that can cause a remarkable variation, which is not limited to body weight and fat distribution, but also extends to include the liver blood transfusion, transporters, and DMEs (Gandhi et al., 2004). Pharmacokinetically, sex-based differences consider the variation in process of absorption, distribution, metabolism and excretion (ADME). However,



metabolism variation is thought to be the major factor that underlies sex differences in pharmacokinetics since they affect DMEs expression level in both Phases I (CYP450s) and Phase II (UGTs, SULTs, and GSTs) (Waxman and Holloway, 2009). Expression of specific individual CYP isoform based on sex was noticed in laboratory animal models such as mice, rat, and human. An early genomic study involving male and female human livers revealed more than 1300 genes, whose mRNA expression is sex-dependent, among these, 40 genes were linked to ADME functions including: CYP1A2, CYP3A4 and CYP7A1 that show a female bias; and CYP3A5, CYP27B1, and UGT2B15 showing male bias (Zhang et al., 2011). Further experiments, conducted in rat and mouse liver models, have resulted in the identification of more than 1000 genes whose expression is significantly affected by sex (Waxman and Holloway, 2009). Proteomics approaches have also been used to involved evaluate sex-differences of CYP450 isoforms using rat and human liver microsomes (HLM) models (Nisar et al., 2004b, Shrivastava et al., 2013a).

Overall, males of human being show a higher rate of clearance of drugs compared to females and therefore, they are less likely to experience adverse drug effects. For example, paracetamol clearance rate is 22% higher in the former due to the high rate of glucuronidation (Miners et al., 1983). This despite some CYP isoforms being expressed in higher amounts or activities in females than occurred in males such as; the predominant CYP3A4 and CYP2A6 respectively (Zanger and Schwab, 2013).

Despite the numerous interspecies variations in the expression, activities, and inducibility of CYP enzymes, mouse as an experimental model has been considered the most similar to human CYP profile (Guengerich, 1997).

However, more CYP450 gene superfamilies were sequenced in mouse (102 genes) as compared to humans (57 genes) (Nelson et al., 2004). Importantly, 36 orthologous pairs of CYP genes from various families (e.g. 1A, 1B, 2B, 2E, 2C, 2D, 4B, and CYP27A) were identified as having the potential for similar or identical functions in both mice and humans. For instance, the selective CYP1A2 inhibitor, furafylline, demonstrated similarities in the inhibition profiles of mouse and human liver microsomes, which indicates a corresponding CYP1a2-metabolism in the mouse as in human (Bogaards et al., 2000).

Cytochrome P450 isoform 1a2 and 2e1 in mouse have demonstrated the direct and meaningful species extrapolation to human. CYP2E1, for example, exists as a single isoform in both human and mouse exhibiting a significant similarity of catalytic specificity (Guengerich, 1997). The similarity of CYP1A2 and CYP2E1 sequence between human and mouse (homologues) are shown in Figure 1.5, including the predicted specific-isoform unique peptides of each isoform.



### 1.3.2 Drug toxicity in the liver

Toxicity of drugs is the main reason of drug failing at various stages during drug development (clinical trials), regardless whether the drug is active or not. Moreover, drug toxicity is also responsible for drug withdrawal from the market (Hay et al., 2014). In general drugs might exert their toxicity due to:

- (i) Mechanism-based drug and target interaction “on-target”, for example, drugs targeting the p38 MAP kinase in rheumatoid arthritis (Hammaker and Firestein, 2010)
- (ii) Unrelated drug-target interaction “off-target”, where drug interacts with one or more unintended targets, resulting in adverse effects on the function of the organ. The main example on off-target is the induction of CYP3A4 and CYP2B6 by phenobarbital (Chu et al., 2009).
- (iii) Biotransformation of drug into reactive intermediates “bioactivation” due to the activity of Phase I enzymes (such as CYP450’s) that are capable of attacking cellular macromolecules, such as those products of paracetamol metabolism.

In addition, other reasons include hypersensitivity and immune response reaction (such as allergic reactions). For idiosyncratic reactions, it has been found that about 35% of known drugs were active against more than one target (Paolini et al., 2006).

Toxicity from drugs or xenobiotics can be manifest in any organ. However, because the liver is the major site in the body responsible for the metabolism of drugs, it is particularly susceptible to toxic insult. Therefore, liver is a useful

organ to provide early indications of drugs toxicity. Drug toxicity may injure the liver causing what is known as drug-induced liver injury (DILI). The mechanism of DILI is complex and can be as a result of:

(i) Drug itself (off-target), by affecting the expression level of drugs metabolizing enzymes, CYP450's, through either inducing or suppressing them, which may lead to drug-drug interaction.

(ii) Formation of highly reactive intermediates such as; Reactive oxygen species (ROS). Indeed, ROS attacks the cellular macromolecule components (DNA, proteins or lipids) causing liver injury (Srivastava et al., 2010, Dizdaroglu, 1998).

DILI can be classified histologically as the following: (i) acute or chronic hepatitis/cholestasis, (ii) zonal or non-zonal primary hepatic necrosis, (iii) reversible hepatic changes such as steatosis, glycogen accumulation, or centrilobular hypertrophy due to CYP450 enzyme induction, (iv) pre-neoplastic/neoplastic patterns of hepatic injury, (v) mixed histological patterns due to a combination of lesions, and (vi) non-specific changes that are secondary to other systemic and metabolic diseases (Ramaiah, 2007).

#### **1.3.2.1 Chemotherapy induces liver toxicity**

Although chemotherapeutic drugs are one of the best choices for killing cancer cells, they can target normal cells that are actively growing and dividing, causing unpleasant transient side effects such as; nausea, vomiting and hair loss (Debatin, 1997). Moreover, chemotherapy drugs may cause reversible and irreversible side effects such as liver injury. Chemotherapy-induced liver

injury (CILI) can be manifest in the changes of liver parenchyma such as; steatosis and chemotherapy induce steatohepatitis (CASH) (Ramadori and Cameron, 2010, Fong and Bentrem, 2006). Furthermore, CILI can result in chemotherapy treatment failure and culminate in serious liver damage and death. CILI may occur due to many causes including the anti-cancer drug itself “intrinsic” or for an idiosyncratic reason such as liver fibrosis prior long methotrexate administration (Fontana, 2014). However, the formation of ROS metabolites is thought to be the leading cause of CILI (Lim et al., 2010). Accumulation of toxins, like ROS in the liver, would lead to adding more stress on the liver’s filtering function. If toxins accumulate in the body at a higher rate than the liver capacity to process them, liver damage will result. Therefore, employing advanced laboratory methods such as quantitative proteomics on animal models of liver injury induced by chemotherapy can provide a predictive insight interpretation about the deleterious effects of chemotherapeutic drugs and the influence of DMEs on toxin formation in the liver (Van Summeren et al., 2011).

### **1.3.3 Proteomics in drug analysis**

Proteomics has been involved in different aspects of drug studies, as the protein is the cellular functional unit that implements a response to any stimuli such as, drugs administration or environmental xenobiotics. Two major terms are contributed to the application of proteomics in drugs investigation: Toxicoproteomics and Pharmacoproteomics.

Toxicoproteomics was first described by Wetmore (Wetmore and Merrick, 2004), which specifically concerns understanding the undesired effects of

xenobiotics. The term “Toxicoproteomics” has been defined as using proteomics to identify critical proteins and pathways in biological systems that are affected by and respond to the toxic effects of xenobiotics (Ge et al., 2007). However, identifying the way that a drug interact with protein is a crucial point that may help in determining the mechanism of drug’s action and toxicity (Van Summeren et al., 2012). Nevertheless, toxicoproteomics is not limited to drugs analysis, as a matter of fact, many studies have been performed concerning chemical, metal and environmental toxins in acute or long-term exposures (Rabilloud and Lescuyer, 2015). For examples, investigating the molecular mechanisms of toxicity from exposure to polyfluorinated compounds using different toxicoproteomics approaches and quantitative toxicoproteomics to investigate the toxic/carcinogenic effect of the potent carcinogen, benzo[a]pyrene, using toxic and sub-toxic doses (Hansmeier et al., 2014, Kalkhof et al., 2014).

On the other hand, pharmacoproteomics is a comprehensive term dealing principally with all aspects of drug administration. Thus, the eventual aims of pharmacoproteomics study include: (i) identification and verification of drug targets, (ii) illustration of efficacy and toxicity mechanism of action of drugs at molecular level and (iii) development of protein biomarkers and assay can be used for assessment of drug in pre-clinical and clinical stages (Zolla, 2008, Hess, 2013).

In order to understand the molecular mechanisms of drug’s efficacy and toxicity, cells or organisms are exposed to either therapeutic or lethal dose of the drug, depending on the type of study, to induce a significant cellular response. By means of proteomics, the proteome of the exposed sample is

then compared to the control proteome to find out the regulated proteins (down-regulated or up-regulated) in the treated sample.

Several pharmacoproteomics studies have been conducted to investigate and understand the mechanism of toxic effects of drugs in pre-clinical and clinical phases of drug development. However, proteomics approaches have also been applied for released drugs (i.e. drugs on the market). For examples, the *hypoglycaemic* drug; “troglitazone” was withdrawn from the market due to its unacceptable idiosyncratic hepatotoxicity (Lee et al., 2013b). Moreover, proteomics has been used to investigate the possible hepatotoxic mechanisms of acetaminophen, cyclosporine A and amiodarone (Van Summeren et al., 2013). Despite the huge use of proteomics in drugs analyses, the reputation of proteomics was highlighted by studying the mechanism of resistance to anti-cancer drugs and the mechanism of their induced toxicity. The high importance of such studies is owing to the non-selectivity of anticancer drugs, which might harm normal cells and the complexity of the molecular mechanisms of their toxicity. Examples of such pharmacoproteomics studies on anti-cancer drugs are shown in Table 1.2.



**Table 1-2. Proteomics studies for some anti-cancer drugs.**

<b>Drug</b>	<b>Model</b>	<b>Species</b>	<b>Organ/cells</b>	<b>Proteomics approach</b>	<b>Reference</b>
<b>Cisplatin</b>	<i>In vitro</i>	Human	Hela	SILAC	(Chavez et al., 2011)
	<i>In vitro</i>	Rat	Hepatocytes	Shotgun, free label	(Cho et al., 2012a)
	<i>In vitro</i>	Human	Ovary mitochondria	Shotgun, spectral counts	(Chappell et al., 2012)
	<i>In vitro</i>	Human	Neuroblastoma cell line	2D-DIGE/ MALDI	(D'Aguanno et al., 2010)
<b>Doxorubicin</b>	<i>In vitro</i>	Human	HepG2	2D-DIGE/MALDI	(Hammer et al., 2010)
	<i>In vitro</i>	Rat	Isolated heart	2D-DIGE/ MALDI	(Gratia et al., 2012)
<b>Bortezomib</b>	<i>In vitro</i>	Human	Myeloma	iTRAQ shotgun	(Uttenweiler-Joseph et al., 2013)
<b>5-fluorouracil</b>	<i>In vitro</i>	Human	Colon cancer cell line	SILAC	(Marin-Vicente et al., 2013)
<b>Vincristine</b>	<i>In vivo</i>	Mouse	Xenograft	2D-DIGE/MALDI	(Verrills et al., 2006)

- Different proteomics approaches were used such as; 2D-DIGE: Proteins were separated by gel in two dimensions, then identified using PMF (peptide mass fingerprint) approach
- Shotgun: Peptides were separated using chromatographic techniques then identified by suitable MS
- iTRAQ: Isobaric tag for relative and absolute quantitation for digested peptides followed by MS-quantitative proteomics
- SILAC: Stable isotope labelling by amino acids in cell culture followed by MS-quantitative proteomics
- Label-free: Proteins quantitation based on measurement of the ion current corresponding to their peptides
- Spectral count: Proteins quantitation through the spectral counting of their independent peptides appearing in the MS/MS analysis.

### 1.3.4 Liver proteomics for studying CYP450s

As stated before, liver is a pivotal organ that is responsible for many of critical functions like drugs metabolism and detoxification. Therefore, the Human Proteome Liver Project (HLPP) was initiated in 2002, to generate a comprehensive protein atlas of the human liver, which can help in understanding the molecular functions of liver proteins (He, 2005). Liver, as a model, has been considered for various drug investigation experiments, however, in pre-clinical drugs development, although, *in vitro* models reduce the number of animals used in pre-clinical trials, researchers don't prefer employing it, as it lacks sensitivity and can generate false-negative results, additionally, *in vitro* models showed variation in DME expression and consequently different response to toxin compound450 (Suter et al., 2011, Hartung, 2009, Uetrecht, 2008).

Currently, most of the available data concerning CYP450s expression were derived from DNA and mRNA-based experiments (Shrivastava et al., 2013a). However, studying the DNA and mRNA provides insufficient information about the expression and activity level of individual CYP450 enzyme, since the correlation between mRNA level, protein levels and enzyme activity for membranous proteins in general and CYP450 isoforms, in particular, were poor or even negligible (Williamson et al., 2011). In the previous study by Ohtsuki and colleagues revealed a poor correlation between mRNA and protein levels of various drug-metabolizing enzymes (CYP450 and UGT) and transporters (Ohtsuki et al., 2012). Western blot (immunoblotting) is another approach used for CYP450 enzyme detection, although it is a very sensitive method, antibodies are not available for all CYP isoforms. In addition, as result

of highly sequence homology within CYP subfamilies, few of the available antibodies can distinguish between closely related isoforms (cross-reactivity). For example, neither polyclonal nor monoclonal antibodies can distinguish CYP2B1 and CYP2B2 (MacLeod et al., 2013).

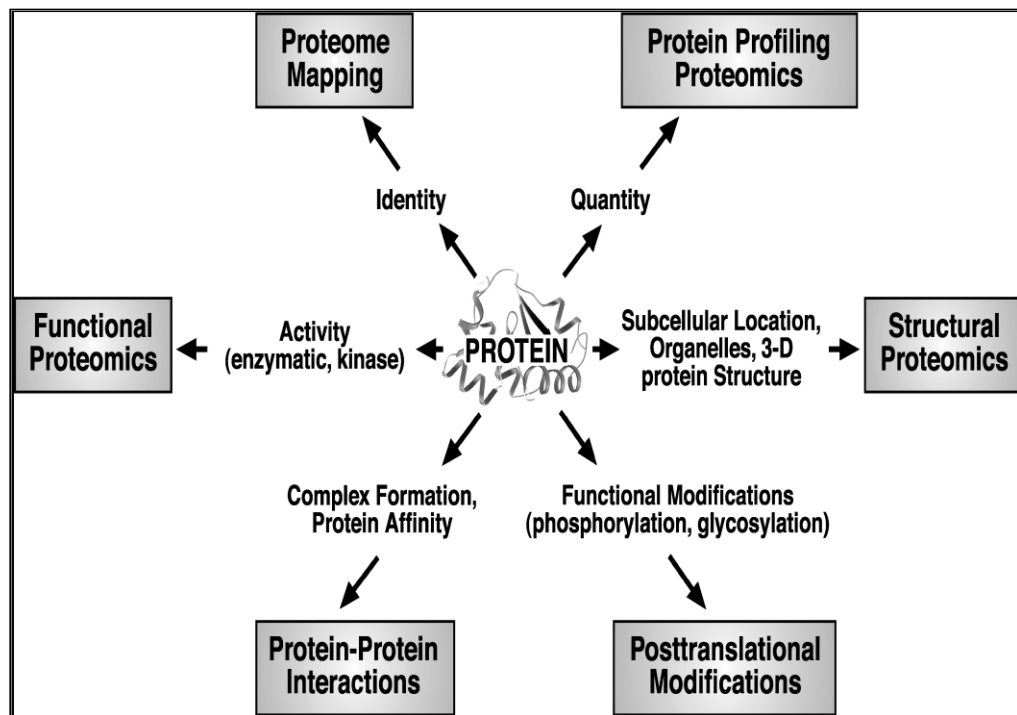
## **1.4 Proteomics**

### **1.4.1 Protein, proteome, proteomics**

Proteins are the functional units in the body, which are composed of amino acids connected together by peptide bonds. Proteins are pivotal molecules that play an important role in the formation of the cell architecture as well as in metabolic processes, cell motility, protein synthesis and mitosis. Proteins play a main role in disease progress and in response to stimuli, for example, drug administration and environmental xenobiotics (Mesri, 2014).

The term “proteome” was first proposed in 1995 by Wilkins et al. (Wilkins et al., 1995) to describe that “**PROTE**in is complementary to the gen**OME**”, meaning the complete set of the proteins expressed by the cell. Whereas Proteomics is the study of protein structure, function, expression, localization, and interactions (Bateson et al., 2011). Proteomics involves the integration of a number of technologies with the aim of identification and quantitation of the protein complement expressed by a biological system in response to various stimuli or to particular physiological or pathophysiological conditions. In general, a proteomics approach can be used for (i) protein profiling, (ii) comparative expression analysis of two or more protein samples (biomarkers identification), (iii) study the protein-protein or protein-drug interaction and (iv)

for the localization and identification of post-translational modifications (Figure 1.6) (Chandramouli, 2009).



**Figure 1-6. Disciplines of proteomics research, including the aspects of proteomics and their role in proteins analysis. (Wetmore and Merrick, 2004).**

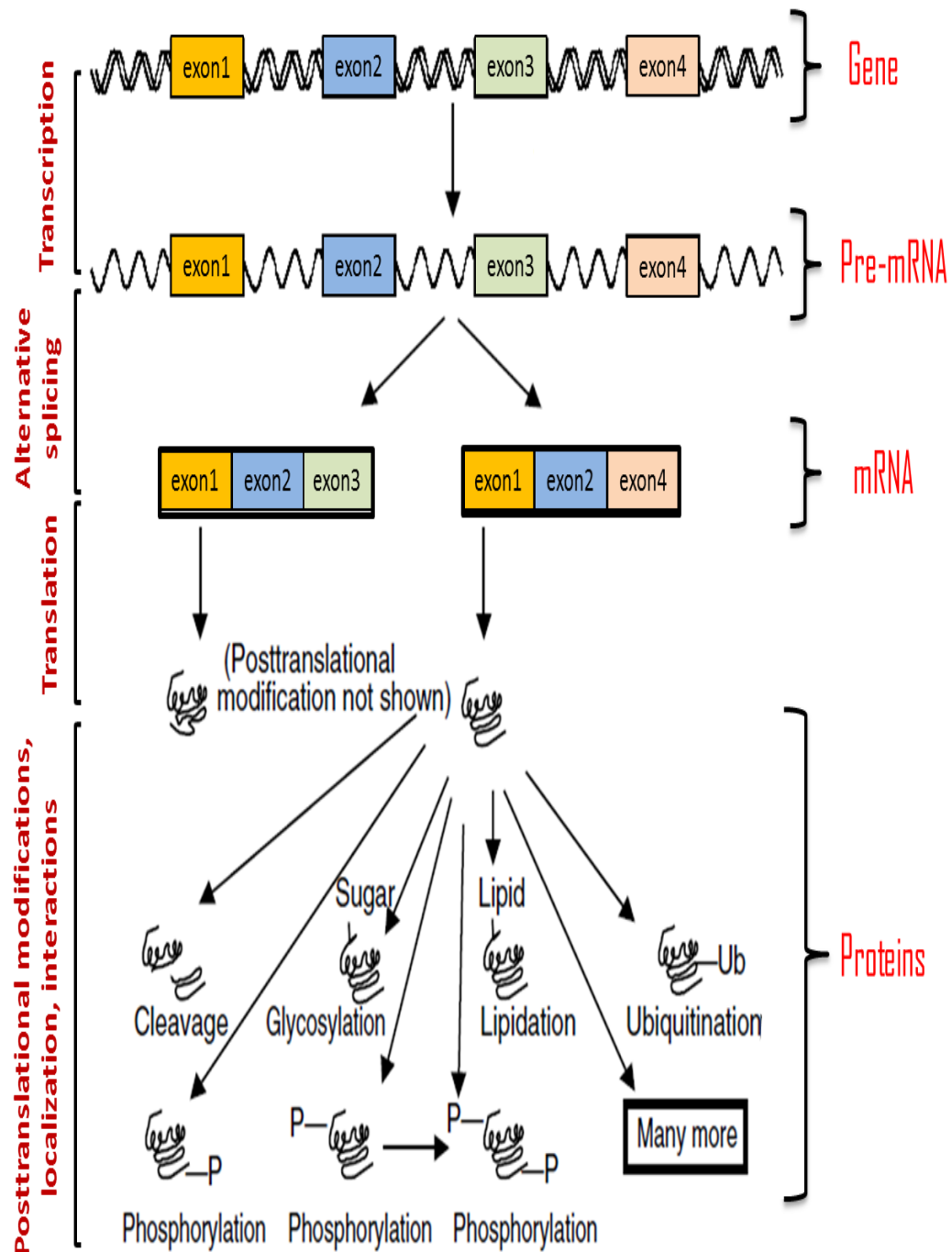
Proteomics is a key technique that is able to correlate those proteins involved in the progress of diseases, since most diseases are expressed at the level of protein abundance or mutation, leading to the identification of new biomarkers that can be targeted for diagnosis or treatment of diseases.

### **1.4.2 Proteomics vs. genomics**

Proteins are the active agents in the cells, tissues, and organs and have an essential role in determining the phenotype of an organism. Even if all the organisms have one unique genome, the proteome of the organism can vary and create different phenotypes for the organism (Diz et al., 2012). The challenge of proteomics versus genomics resides in the complexity of protein

chemistry and multiple potential post-translational functional modifications contrasting with the unique nucleotide complement and sequence upon which genomics relies (Smejkal, 2012). The human genome is estimated to be composed of approximately 21,000 protein-coding genes, which generate around 500,000 or more distinguishable functional proteins, where each single gene may encode for single or multiple proteins (Smejkal, 2012). This is due to the differential splicing and translation of many genes and numerous post-translational modifications of proteins. Figure 1.7, illustrates the complexity of the cellular proteome.

An advantage of analyzing the proteome rather than the genome is that proteomic changes are dynamic in contrast to genomic which are static; thus, proteomics can reflect the physiological and pathophysiological conditions much more accurately. This enables close monitoring of changes in the state of cells, tissues or organism over time. Another advantage is the dynamic range of concentrations of proteins since one cell can contain one to more than 100,000 copies of each protein, which is not reflected in genomic information (Chandramouli, 2009, Kolch et al., 2005). Furthermore, proteomics can identify the post-translational modifications of proteins which have profound effects on the biological function and their cellular localisation (Page et al., 1999, Parekh and Rohlf, 1997). In addition, protein functionality often demands specific protein-protein interactions forming functional protein complexes (Dziembowski and Séraphin, 2004).



**Figure 1-7. The complexity of proteome is much more than its corresponding genome. As a result of the alternative splicing, one gene produces multiple pre-mature mRNA transcripts and thus generating multiple proteins. After translation step, a myriad of post-translational modifications can create additional distinctions in the number and natures of protein form. Many different forms can be produced due to post-translational modifications, eight of which are shown. The figure is adapted from Peng et al, (Peng and Gygi, 2001).**

### **1.4.3 Proteomics aspects**

Proteomics covers a number of different aspects of protein function, including:

(i) structural proteomics that maps out the structure of proteins to help identify protein localization and the way they interact with the ligand. This is achieved using technologies such as X-ray crystallography and NMR spectroscopy. (ii) Expressional proteomics also called differential protein expression, which measures changes in protein expression in related samples, such as diseased versus healthy tissue. A protein found only in a diseased sample may represent a useful drug target or diagnostic marker. Proteins with similar expression profiles may also be functionally related. Technologies such as 2D-gel electrophoresis and mass spectrometry are used for these studies. (iii) Functional proteomics is concerned with determining protein-protein interactions and their impact on protein function, both normal and abnormal. Technologies such as affinity purification, mass spectrometry, and the yeast two-hybrid system are particularly useful for these studies (Graves and Haystead, 2002, Ning et al., 2011).

#### **1.4.3.1 Proteomics in biomarker discovery**

A biomarker is defined as a characteristic and measurable indicator of normal biological process, particular pathophysiological or physiological conditions, or pharmacologic response to a therapeutic intervention (Atkinson et al., 2001). Biomarkers play an important role in drug discovery and development as well as in understanding the causes and progression of the disease.

Mass spectrometry-based proteomics has been shown as a successful means for biomarker screening in clinical and pathological conditions. Nowadays,

proteomics is employed significantly in identification biomarkers for several diseases such as Alzheimer's, asthma, and chronic obstructive pulmonary disease (Galasko, 2005, Verrills et al., 2011). In oncology, proteomics has been applied to understand the pathology of cancer, implement tumor monitoring and identify novel targets for cancer therapy as well as for prognostic and diagnostic purpose. For examples, identification of urinary S100A9 protein as a potential biomarker for early detection of hepatocellular carcinoma and PGAM1 proteins as a therapeutic potential target for urothelial bladder cancer (Huang et al., 2015, Peng et al., 2016). Certainly, identified biomarkers have to be validated to make sure that these biomarkers are confidently associated with the defined biological statistics and can be reproducibly performed (Issaq and Veenstra, 2008).

## **1.5 Proteomics workflows**

There is more than one design for proteomics experiment workflow depending on the aim of the study. However, a typical quantitative proteomics workflow consists of biological sample preparation, protein extraction, protein separation, proteolytic degradation, peptide separation, mass spectrometer analysis and database analysis. All the methods used in different stages are linked very closely with each other, for example, a suitable MS analysis method should be considered based on the sample preparation and separation method.



### **1.5.1 Sample preparation**

The first step in proteomics workflow is sample preparation, which includes at least three steps: cell/tissue disruption, protein solubilisation, and removal of interfering substances. Extracting high quality and adequate amounts of proteins from sample for proteomics analysis should be as simple as possible to maximize reproducibility and reduce yield loss. Proteins can be extracted from many different sources, such as cultured cell lines, tissues, body fluids, etc.

Protein extraction is achieved by three methods; mechanical, chemical and enzymatic, individually, more often in combinations. Chemical based extraction methods using detergents or organic solvents have become very popular, due to their ease of use, low cost and new protocols with proven efficiency. Detergent-based extraction methods involve cell membranes disruption, breaking lipid-protein interactions and solubilising proteins. However, detergents remain a significant challenge in MS-based proteomics study (section 3.1.1), as they generate background signals that may suppress peptide-derived signals or contaminate the MS source (Wiśniewski et al., 2009). Therefore, detergent concentration needs to be optimised, or removed during protein preparation and before MS analysis. Examples of chemical methods are illustrated in Table 1.3.

**Table 1-3. Different groups of chemical agents used for cell membrane disruption and proteins solubilisation with their applications**

<b>Chemical method</b>	<b>Materials</b>	<b>Applications</b>
<b>Detergent</b>	<ul style="list-style-type: none"> <li>➤ Ionic: SDS</li> <li>➤ non-ionic: Triton X-100, 114</li> <li>➤ zwitter-ionic: CHAPS</li> </ul>	Bacterial cells, plants, animal tissues
<b>Organic solvents</b>	<ul style="list-style-type: none"> <li>➤ Acetonitrile</li> <li>➤ Methanol</li> <li>➤ Isopropanol</li> </ul>	Bacterial cells, plants, animal tissues
<b>Organic acid</b>	<ul style="list-style-type: none"> <li>➤ Formic acid</li> </ul>	Animal tissues

Mechanical based extraction methods have shown a notable ability to disrupt the tissues or cells, allowing the rapid release of the intracellular proteins. These proteins are released into a harmless buffer that can keep the activity of the protein of interest (Carpentier et al., 2008). Mechanical methods (Table 1.4), can be performed using many techniques varying from gentle to harsh, depending on the type of sample, the stability and cellular location of proteins required to be investigated (Carpentier et al., 2008). Mechanical methods include:

### **1. The freeze-thaw method**

The freeze-thaw method is the simplest mechanical method that is used commonly to lyse cells from mammalian tissues and bacteria. The technique is based on freezing a suspension of cells and then thawing it back at room temperature. For freezing phase, liquid nitrogen or dry ice-ethanol bath can be used. During the freezing-thawing cycles, the cells size is increased where ice crystals are formed that eventually breakdown in the thawing phase.

Repeating the cycle is essential for efficient lysis, which lengthens the process time. However, the freeze-thaw method has been shown to efficiently release 40%-90% of over-expressed recombinant protein located in the cytoplasm of *Escherichia coli* bacteria (Johnson and Hecht, 1994).

## **2. Cryo-pulverization**

In this method, the sample is frozen in liquid nitrogen making it fragile and easily fractured. The sample is then crushed using a pre-chilled mortar and pestle to a fine powder. Cryo-pulverization is an effective method that has many advantages of enabling the fine powder to be recovered easily and extraction from large tissues or whole organs. Moreover, due to the extremely low temperature, the cryo-pulverization method is able to extract proteins preserving their activity (e.g. for enzyme activity assays) (Butt and Coorssen, 2006, Ericsson et al., 2007).

## **3. Sonication method**

Sonication is the most common process for cells disruption. Due to the difficulty in maintaining the low temperature and the long sonication time to achieve full lysis for cells, this method is preferably used for relatively small quantities. Sonication disrupts cells by using high-frequency sound waves, releasing proteins from tissue that has broken. A sonicator typically delivers sound waves using a vibrating probe submerged in a liquid suspension of cells. Varying powers can be applied depending on the cell type. The sonication run is conducted in multiple short bursts using an ice bath for the sample in order to prevent excessive heating and protein damage. Sonication method is adaptable for different sample volume, which is used commonly for cells suspension (Goldberg, 2008, Vilkhov et al., 2011).

#### **4. Liquid-based homogenization method**

Liquid-based homogenization is used mostly for small volume samples, usually cells and sections of tissue or whole organs in the buffer. It is based on sharing the forces between cells or tissue suspension through a narrow space of glass tube. Generally, there are different types of liquid homogenizers available come up with diverse sizes to adapt a range of sample volumes; moreover, they are inexpensive, easy to use, and clean (von Hagen, 2011).

Dounce homogenizer is an example of the liquid homogenizer, which consists of a glass pestle shaped to fit a rounded glass tube. The sample suspension is added to the glass tube then gradually pressing the pestle on the sample manually. The number of strokes and the speed based on the size and type of sample (Simpson, 2010). However, the French press is another example on liquid homogenizer that applies high pressure automatically. It has been involved extensively in protein extraction from blood cell disruption and minced animal tissues (von Hagen, 2011).

#### **5. Electrical homogenizers or blenders**

Electrical homogenizer or blender is considered as the toughest method for protein extraction. Large solid tissues, such as liver and those organs with extensive fibrous connective tissues, are ground and dispersed by blades in electrical homogenizers which chilled on ice (Bodzon-Kulakowska et al., 2007).

**Table 1-4. Different groups of mechanical methods used for cell membrane disruption with their applications.**

<b>Mechanical methods</b>	<b>Materials</b>	<b>Applications</b>	<b>General procedures</b>
<b>Freezing and thawing</b>	Liquid nitrogen	Bacterial cells, plants, animal tissues	Rapidly freeze cell suspension using liquid nitrogen and then thaw.
<b>Sonication</b>	Sonicator to produce ultrasonic waves	Cell suspensions.	Sonicate cell suspension in short bursts to avoid heating. Cool on ice between bursts.
<b>Grinding (pulverization)</b>	Pre-chilled mortar and pestle	Solid tissues, microorganisms	Tissue or cells are normally frozen with liquid nitrogen and ground down to a fine powder.
<b>Homogenizing</b>	Electrical homogenizer (blender)	Solid tissues or Cell	Rotating blades grind and disperse cells and tissues into small pieces using chilled buffer
	a liquid homogenizer (Dounce)	Solid tissues or Cell	Tissue suspensions are sheared by forcing them through a narrow space
<b>High pressure</b>	Chilled French press	Microorganisms with cell walls	Applying pressure on cell suspension

In order to characterise the whole cell proteome, proteins must be solubilised during extraction. Proteins are often found in complexes with membranes, such as nucleic acids or other proteins. Some proteins form non-specific aggregates and precipitate when removed from their normal environment. Different treatments and conditions are required to solubilise different types of protein samples depending on the type of protein extraction, protein concentration and the solubilisation method (Pelkonen et al., 1974). For

example, detergents such as Triton X-100 and sodium deoxycholate can be used at low concentrations for membranous proteins solubilisation.

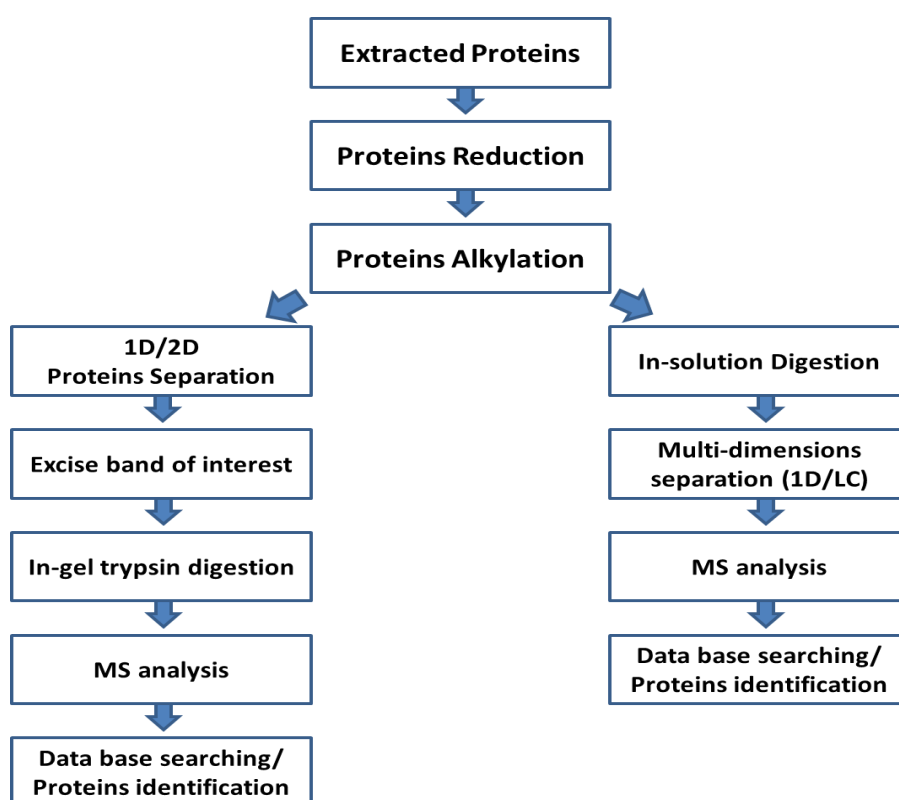
During the process of protein extraction, cellular endogenous proteases are released, which may degrade cellular proteins rendering them unsuitable for subsequent analysis (von Hagen, 2011). In order to inactivate the enzymes, specific protease inhibitors (e.g., EDTA, chloromethyl ketone, or benzamidine) are added to prevent protein degradation. Moreover, samples are kept at low temperature, typically 4°C, to reduce proteolytic activity and since most mechanical methods release heat, affecting protein stability and activity. After protein extraction, contaminants such as; salts, nucleic acids, detergent, polysaccharides or lipids are removed by a desalting method such as dialysis or protein precipitation (Feist and Hummon, 2015).

Extracted proteins are further reduced to cleave inter- and intra disulphide bonds crosslink within and between protein subunits and then alkylated to prevent reforming. The most common reducing agents are; beta-mercaptoethanol, dithiothreitol (DTT), tributylphosphine (TBP) and tris-(2-carboxyethyl)-phosphine (TCEP). Iodoacetamide, acrylamide derivatives, and vinyl pyridines are used as common alkylating reagents (Bai et al., 2005, Righetti, 2006, Darie et al., 2004).

The final step in sample preparation is protein digestion. There are many chemicals and enzymes with different specificities available for protein digestion (further information on ExPASy; [\[au.expasy.org/tools/peptidecutter/peptidecutter\\_enzymes.html\]](http://au.expasy.org/tools/peptidecutter/peptidecutter_enzymes.html)). However, trypsin is the most commonly used protease since it has well-defined cleavage specificity at the C-terminal side of the basic amino acid residues lysine and

arginine unless the next residue is a proline (Olsen et al., 2004). The resulting peptides after trypsin digestion remain charged, which improves their ionization in the MS analysis, and for the most part are in the mass range suitable for MS/MS analysis and protein identification by database searching (Hustoft et al., 2012).

In general, there are two approaches to converting proteins into peptides suitable for MS-based proteomics analysis; in-gel and in-solution methods, depending on the method of protein fractionation (Figure 1.8).



**Figure 1-8. Workflows of in-gel (left) and in-solution (right) digestion and subsequent LC-MS analysis of a protein sample.**

In the in-gel digestion method, the extracted proteins are first solubilised using detergent, and then separated in one or two dimensions (1D/2D) via SDS-PAGE and finally the bands of interest with defined molecular weight are

excised and digested enzymatically with trypsin. On the other hand, in gel-free or in-solution digestion is followed by extensive separation of the resulting peptides using multi-dimensional chromatographic and/or electro-focusing methods, followed by MS analysis and protein identification (Yates et al., 2009).

### **1.5.2 Subcellular fractionation**

The presence of multiple forms of each gene product indicates the diversity generated by the alternative splicing of mRNA and the variety of post-translational modifications that occur upon protein synthesis, altering the physical and chemical properties of proteins (Duan and Walther, 2015). In addition, cellular protein concentrations are extremely wide, ranging from 7 to 10 orders of magnitude, making it difficult to detect the low abundance proteins (Dwane and Kiely, 2011). In order to improve the resolving power of proteomics, pre-fractionation strategies are used to reduce sample complexity and allow detection of less abundance protein.

Since differential centrifugation separates cellular components based on their physical characteristics, it is considered the most effective approach and is compatible with analytical proteomics techniques (Huber et al., 2003).

### **1.5.3 Proteomics approaches in proteins separation**

Protein identification traditionally follows one of two proteomics workflows, which differ based on the way extracted proteins are converted to peptides suitable for mass spectrometry (MS) analysis, as shown in Figure 1.8. Separations are performed prior to MS to simplify the complexity of protein



mixture and improve the information extracted from the proteome. Of the possible methods for proteome separation, gel electrophoresis (1D/2D-GE) and multidimensional liquid chromatography are the two most commonly used methods.

#### **1.5.3.1 One and two-dimensional polyacrylamide gel electrophoresis approach**

The gel electrophoresis-based technique was one of the original protein separation methods used in proteomics studies and can be performed in one- or two-dimensions depending on the complexity of the sample. One-dimensional gel electrophoresis separates proteins based on their molecular weights. Chaotropes (e.g., urea and thiourea) and anionic detergent (e.g., sodium dodecyl sulphate (SDS)) are used in sample and gel preparation to unfold proteins, ensuring they are linearised and migrate based on their relative molecular weights (Laemmli, 1970). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is an excellent method to separate proteins and compare samples qualitatively throughout sample preparation and to assess the consistency of enriched fractions. However, it is also an effective low-resolution preparative method for fractionating hundreds of proteins in a single gel (Thakur et al., 2011). It has been used very effectively as an enrichment step for isolation of cytochrome P450s, all of which have similar molecular weights (approximately 55 to 60kDa) from liver microsomes (Nisar et al., 2004b, Sutton et al., 2010). SDS-PAGE can also be used in conjunction with blotting methods, for example, western blot or immunoblot, for specific protein detection using antibodies (Garfin, 2009).

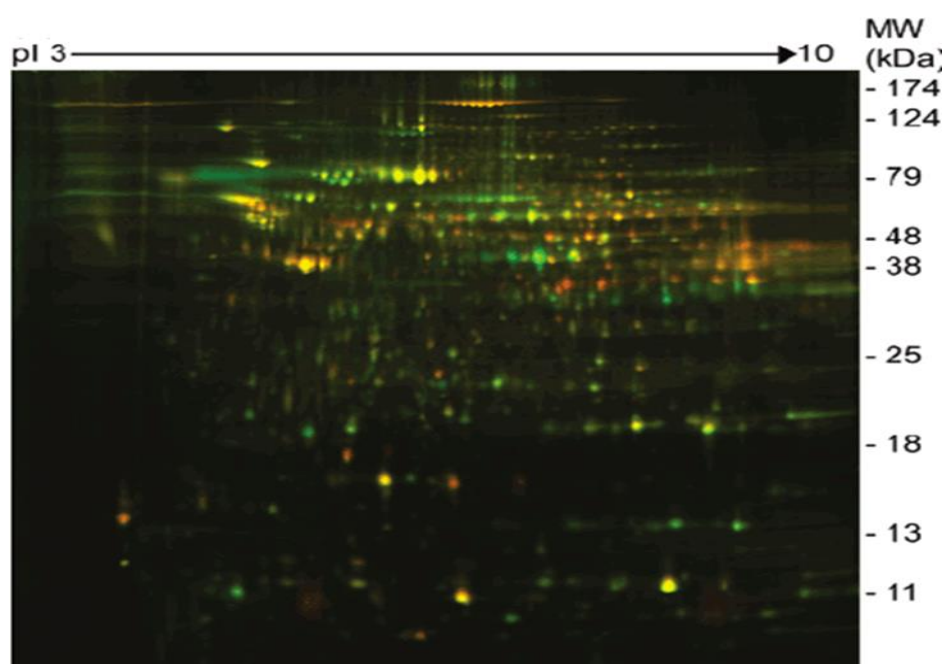
Protein charge can also be used as the basis of separation using isoelectric-focusing (IEF), which separates proteins based on their isoelectric points (pI). Two-dimensional gel-based separation (2D-PAGE) was first introduced by O'Farrell in 1975, for visually profiling total protein extracts, but it was not used until the advent of proteomics in the early 90's that 2D gels proved their full potential as a two-step separation of proteomes prior to in-gel-trypsin digestion and mass spectrometric analysis (Figure 1.9) (O'Farrell, 1975). The resolution power of these two orthogonal separation techniques for proteins results in separating each protein isoform with specific pI and molecular weight coordinates, while the volume of the spot is correlated to the protein expression in the sample (Natale et al., 2012).

#### **1.5.3.2 In-gel visualisation and differential gel electrophoresis**

Once proteins have been separated by electrophoresis, staining techniques can be used to visualize proteins. Examples of staining dyes are: Coomassie blue (Neuhoff et al., 1988), silver stain (Winkler et al., 2007), zinc-imidazole stain (Fernandez-Patron et al., 1998), or fluorescence staining or labelling such as, ruthenium bathophenanthroline disulfonate (Sypro Ruby) and Deep Purple (Lunardi, 2001, Bell and Karuso, 2003). Gel staining can be carried out with the aim of evaluating the efficiency of proteins separation and/or for in-gel digestion for peptide mass fingerprinting (PMF). In the case of PMF, spots of interest are identified, excised from the gel and trypsin digested for the MS analysis (Klose and Kobalz, 1995).

Two-Dimensional Differential Gel Electrophoresis, (2D-DIGE) is a variation of 2D-PAGE for differential mapping of two proteomes for comparative

proteomics analysis. Two or more protein samples are labelled with different fluorescent dyes (known as CyDyes: Cy2, Cy3, and Cy5) prior to 2D-PAGE (Marouga et al., 2005, Westermeier and Scheibe, 2008). The ultimate aim of using the fluorescent dyes is to identify those protein spots uniquely stained in each biological conditions (normal versus diseased or control versus treated) and then identify these protein expressions by in-gel digestion and peptide mass fingerprinting (Figure 1.9).



**Figure 1-9. Two-dimensional gel electrophoresis for proteins from hepatocellular carcinoma sample (Cy3, green) and non-cancer sample (Cy5, red). Proteins (50 µg) were subjected first to IEF in an IPG strip (24 cm) 3-10 pH range, and then SDS-PAGE was performed on 12.5% polyacrylamide gel in 2D. Cyanine dye-labelled protein gel was fluorescently scanned directly for gel visualization (Lee et al., 2005).**

There are a number of advantages that made 2D-PAGE widely used in early proteomics studies. First, 2D-PAGE has the high-resolution power with the potential to visualize over 2000 spots corresponding to over 1,000 proteins (Rabilloud et al., 2010). Second, it is able to visualize the proteome map more

effectively than chromatography-based separation; enabling identification of protein isoforms and post-translational modifications such as glycosylation and phosphorylation (Lopez, 2007).

However, 2D gel-based method has a number of disadvantages such as; inability to resolve proteins with very high or very low molecular weight and very acidic or very alkaline pIs (Koga, 2008), narrow dynamic range which limits sensitivity to detect low abundance proteins (Chevallet et al., 2008), limited capability to resolve membranous proteins (e.g. Cytochrome P450 enzymes) (Kanaeva et al., 2005, Rabilloud, 2009), and poor reproducibility (Chevalier, 2010). Reducing the sample complexity through subcellular pre-fractionation (Pasquali et al., 1999) or using narrow range of pI strips (IPGs) (Dépagné and Chevalier, 2012) can overcome dynamic range issues.

#### **1.5.3.3 Chromatography-based separation (multidimensional based proteins separation)**

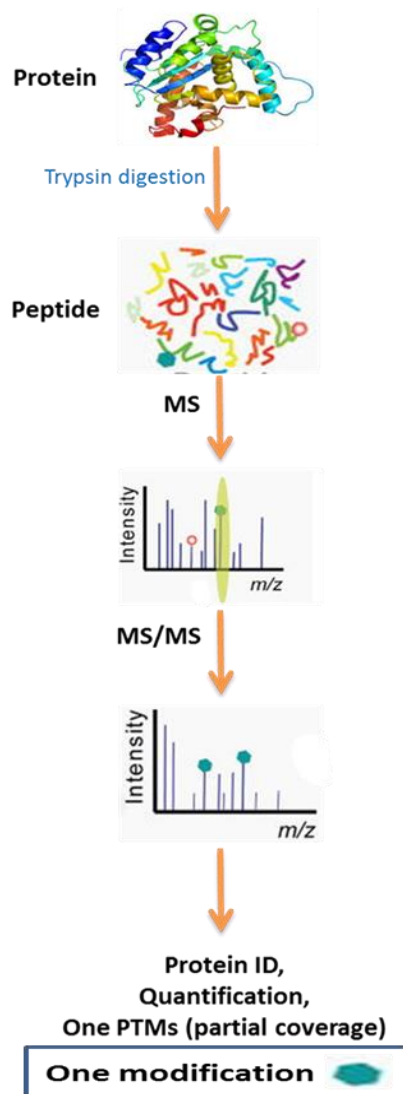
Protein or peptide separation plays an important role in protein identification, where proteome coverage depends mainly on the extent of protein separation to resolve the complex protein mixture and low abundance proteins. Chromatography as a technique can also be used to reduce sample complexity. Here, proteins are usually digested enzymatically into peptides prior to their separation chromatographically.

“Top-down” and “Bottom-up” approaches are two main analytical strategies for proteome separation and identification (Figure 1.10). “Top-down” approach is based on the separation of whole sample intact proteins directly by, for example, high performance liquid chromatography (HPLC) and subsequently

analysed directly by fragmenting the intact protein by mass spectrometry (MS), which provides valuable information about proteins at molecular level and detection of post-translational modifications (PTMs) (Ryan et al., 2010). However, the "Top-down" approach has a limited resolution, requires pure proteins, and in practice is limited to proteins less than 500 amino acid residues (approximately 50 kDa) (McLafferty et al., 2007). Complementary to "Top-down", the "Bottom-up" approach which also named by shotgun or MudPit strategy intends initially to digest proteins into peptides with specific proteases such as trypsin, then reduces their complexity by using multi-dimensional chromatography techniques, such as two-dimensional HPLC (2D-HPLC), prior to analysis by tandem MS (MS/MS). The advantage of the shotgun approach is to reduce the dynamic range of different physio-chemical properties of proteins, such as mass and charge, thereby simplifying the separation and identification by LC-MS system (Washburn et al., 2001a). However, similar to 2D-PAGE, protein inference can be a challenge in shotgun approach; where the identical peptide sequence can be detected in many different proteins or isoforms and cannot be specifically assigned (Zhang et al., 2010). Multi-dimensional proteomics abbreviated as MudPit, is an integration of two or more separation method coupled together to separate either proteins or peptides, which as a consequence enables automation reducing cost and time (Washburn et al., 2001b). Unlike gel-based technique, MudPit is a comprehensive approach able to identify the whole cellular proteome (Abdallah et al., 2012). In MudPit, the first dimension separation approach is used as a preliminary step to reduce the complexity of sample

which is then followed by the second dimension. Proteins/peptides throughout the 1D are resolved to depend mainly on their physio-chemical properties.

### (A) Bottom-up MS approach



### (B) Top-down MS approach

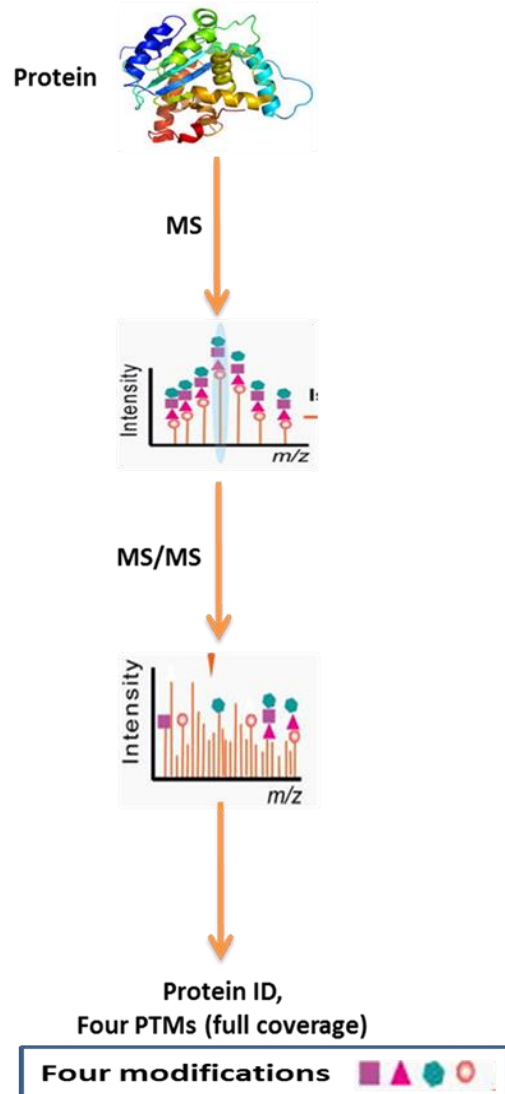


Figure 1-10. In bottom-up MS approach (A), a protein is typically digested enzymatically (i.e. trypsin) into peptides either in-gel or -solution prior to peptides sequencing then protein identification and quantification take place. However, since this approach identifies protein via a small number of peptides (partial coverage), only one modification was determined. In Top-down MS approach, the intact protein (i.e. undigested) is analysed straightforward by MS, where fragmentation can take place for a distinctive protein to locate the modifications sites. However, more modifications (four modifications) were defined since a full coverage was achieved.

The Figure is adapted from (Zhang and Ge, 2011).

#### **1.5.3.3.1 Peptide chromatography**

Peptide separation plays a critical role in comprehensive proteome analysis strategies. It can substantially increase the number and confidence of identifiable components in a proteome. Chromatography-based methods such as ion exchange, hydrophilic interaction, affinity purification, and reverse phase–LC are normally engaged in shotgun proteomics strategy in tandem prior MS identification.

**Ion exchange chromatography (IEC)** takes the advantages of the overall ionic charges of proteins/peptides for first dimension separation. In IEC, analytes with an opposite charge bind to charged functional groups of IEC media, thus, negatively charged proteins are bind to a positively charged group in anionic exchange (AX) chromatography. Conversely, in cationic exchange (CX) chromatography positively charged proteins bind to a negatively charged media. In order to get proteins eluted, a linear gradient of salt concentration buffers at fixed pH is used to competitively displace the anionic or cationic analytes of increasingly high charge states (Di Palma et al., 2012b). Typically, buffer used (such as; ammonium bicarbonate or ammonium formate) should have specific properties. For example, it has to be relatively volatile, with no effect on sample pH, able to elute analytes from the column, and finally MS compatible. The stationary phase in CX is composed of either strong or weak negatively charged residues that allow its binding to the positive moieties of analytes. In contrast, AX is packed with either strong or weak positively charged residues that can bind to the anionic moieties of analytes.

**Strong cation exchange (SCX)** is the most popular choice of IEX prior to RP-LC in MudPit, although strong anion exchange (SAX) is indeed possible. Theoretically, about 29% of human peptides generated after trypsin digestion are not expected to be retained by SAX as they possess either neutral or basic net charge at pH less than 8.5 (Dai et al., 2009). Additionally, SCX is capable of working over a broad range of pH particularly as low as pH 3.0 without losing their negative charges (Di Palma et al., 2012b), since most of the peptides tend to have pIs around pH 4 (Cargile et al., 2004a). However, SAX has a bias to acidic peptides, such as phosphorylated peptides, which makes it superior in their separation and enrichment (Han et al., 2008).

**Affinity purification chromatography (APC)** is a highly selective separation method that involves protein-ligand interactions. In this method, a protein of interest binds by virtue of its specific properties to a stationary phase with an appropriate ligand via reversible biological interactions due to electrostatic or hydrophobic interactions, van der Waals' forces and/or hydrogen bonding (Ayyar et al., 2012). APC method is involved mainly for purification of definite proteins or class of proteins, such as antibody purification (Ayyar et al., 2012). However, in proteomics analysis, APC is used to enrich a particular class of proteins by depleting the high abundant proteins, such as removing albumins from blood serum (de Moraes-Zani et al., 2011).

**Hydrophilic interaction liquid chromatography (HILIC)** was suggested firstly by Alpert in 1990 (Alpert, 1990). HILIC is a normal phase liquid chromatography (NP-LC), where stationary phase is more polar than the mobile phase. HILIC separates proteins based on interactions of hydrophilic amino acid residues of the digested protein with hydrophilic groups on a resin.



Besides peptides, HILIC is frequently used in separating polar/basic compounds separation, such as drugs compounds and their metabolites (Hsieh, 2008). However, the main challenge of using this method is the polarity of the mobile phase that is commonly used to elute polar compounds. The resultant polar compounds can distort the shape of the peak and result in a mismatch between mobile solvent peak and compound solvent peaks (Fritz et al., 2009).

Electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) is a subset of HILIC which separates peptides on the basis of the simultaneous effect of electrostatic forces and hydrophilic interaction, by adjusting pH, salt component and concentration, and organic solvent compositions in the mobile phase. with elution in order of high to low pI and GRAVY values (Alpert, 2008). This affords convenient separations of highly charged peptides that cannot readily be resolved by IEC (i.e. SCX). In addition, phosphopeptides can be isolated selectively from a tryptic digest (Hao et al., 2011).

In contrast with HILIC, **Reversed-phase liquid chromatography (RP-LC)** takes advantage of the hydrophobicity of peptides for separation, where stationary phase contains non-polar residues (Buszewski and Noga, 2012). Analytes such as peptides are separated on the column, by increasing the organic solvent in a linear gradient (Sandra et al., 2008). RP-LC generally is used as the single phase and as the last dimension of multi-dimensional separation prior to MS analysis (Fournier et al., 2007). Moreover, RP-LC is coupled directly to the MS, which gives high resolution, efficiency, reproducibility, and mobile phase compatibility with MS (Shen and Smith, 2002).

Two dimensional-liquid chromatography (2D-LC) is an integration of two-separation methods in tandem, which offers a great improvement in the resolving power over the conventional one dimension. The efficiency of the 2D separation strategy is determined by the alternative selectivity between the separation dimensions and the chromatographic power of the separation systems employed in the different dimensions (Dugo et al., 2008). The power of the orthogonal system of first dimension approach, such as IEC, and a second dimension (i.e. Reverse phase-LC) is being a successful approach that maximizes the extent of peptide dispersion and, thus, influences the number and confidence of identified proteins. SCX in conjugation with RP-LC is the most typical combination used in shotgun approach (Betancourt et al., 2013).

Although 2D-LC approach has proven its ability to overcome sample complexity and the massive differences in protein concentration challenges in Bottom-up, separating abundant proteins from lower abundant proteins by fractionation of cellular proteins into the different compartment (i.e. subcellular fractionation) is another method to increase the proteome coverage analysed by MS. Also, employing a third dimension of liquid separation (e.g. capillary electrophoresis (CE)) has been used recently in Bottom-up approach to achieve total proteome coverage (Zhang et al., 2007).

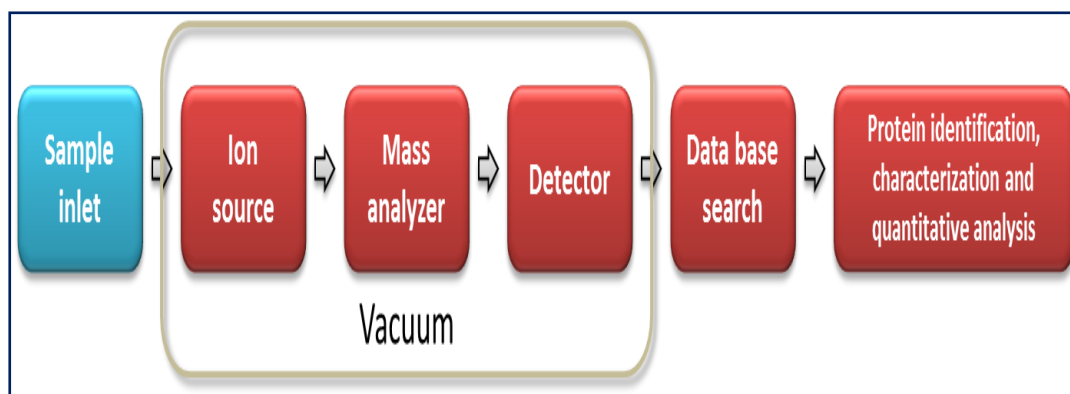
#### **1.5.4 Mass spectrometry**

Mass spectrometry (MS) is an analytical technique that intends to measure the masses of individual molecules after they are converted into ions. Since the first MS was constructed in 1912 (Dempster, 1918), it has been employed in several applications such as; proteomics field, drug discovery (identification of

drugs structure and their metabolites), clinical examination (biomarkers identifications), food safety assessment (food contamination), environmental analysis, space exploration and many others (De Carolis et al., 2014). The prominence of MS has been highlighted in proteins and peptides analysis, where it is capable of measuring molecular weights over 1 million Da, determine protein structure and performs amino acid sequencing. Traditionally, before biomolecular MS, proteins were identified using Edman degradation method that is based on sequencing proteins or peptides from the amino terminus. However, despite being very specific, it was time-consuming and relatively low sensitivity (Edman, 1950).

The advancement of mass spectrometric ionization technology allowed the detection of thermally labile, polar and non-volatile protein/peptide analytes, becoming the dominant analytical method for proteome analysis (Yates et al., 2009). Mass spectrometry nowadays is used routinely in proteome analysis to identify proteins, quantify their levels (relative or absolute) and to characterize protein post-translational modifications (Yates et al., 2009).

Peptides analysis using MS can be conducted in various ways, depending on the MS instrumentation and the way of ionisation. In all cases, the peptide molecules must first be ionized using one of the soft ionizing techniques to produce charged ions (positive or negative) in a gaseous phase. Charged ions are then captured with a mass analyser to separate the ionized analytes according to their mass-to-charge ratio ( $m/z$ ). The mass-to-charge ratio is then determined with a detector. However, typically, MS consists of three essential components: an ion source, a mass analyser and a detector as demonstrated in Figure 1.11.



**Figure 1-11.** The main stages of Mass spectrometric analysis for protein identification are shown. Proteins/peptides sample can be introduced to MS through different means including, HPLC, sample plate or by direct injection for analysis. The ion source is responsible for creating the ionized analytes such as peptides, which are the introduced into the mass analyzer and separated on basis of mass-to-charge ( $m/z$ ). Ionized molecules are detected and counted at each mass-to-charge ( $m/z$ ) value.

#### 1.5.4.1 Ion source

Different techniques have been discovered for sample ionization, such as fast atom bombardment (FAB), chemical ionization (CI), atmospheric pressure CI, plasma desorption (PD), electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (De Carolis et al., 2014). Depending on the nature of sample one of these methods is selected and the soft ionization techniques, with ESI and MALDI, are the ones most commonly used for protein and peptide analysis (Emonet et al., 2010).

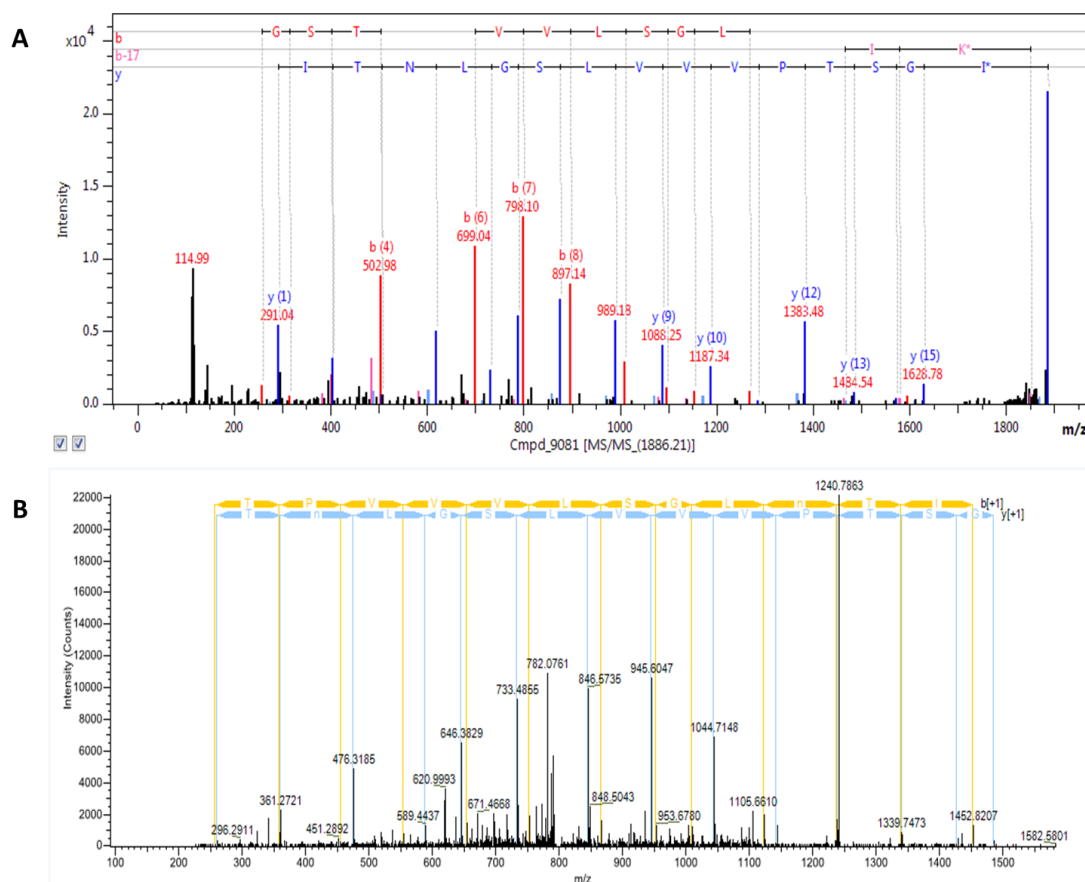
Even though both methods produce charged ions in a gaseous phase, each method is based on a very different principle. ESI changes the introduced liquid sample into very small droplets of solvent-containing analytes, through a combination of voltage, heat, and air (Yates et al., 2009). The continuous loss of solvent results in producing a constant stream of ions. On the other hand, MALDI uses a pulsed laser beam fired at the sample to get it ionized,

after mixing it with a suitable matrix, which is allowed to dry on a conductive surface (Table 1.5) (de Hoffmann and Stroobant, 2007).

**Table 1-5. A comparison between MALDI and ESI ionization methods. Table was adapted from (Cho, 2007, Yang et al., 2007, Seymour et al., 2010, Awad et al., 2015)**

Comparison	MALDI	ESI
<b>Sample status</b>	Solid (Co-crystal with matrix)	Liquid (In solution)
<b>Charge states and adducts</b>	Usually single charge ( $M+H^+$ )	Multiple charges ( $M+nH$ ) $n^+$
<b>Charge polarity</b>	Positive and negative mode	Positive mode
<b>Method of ionization</b>	Laser	Voltage, heat and gas (nebulization)
<b>Power of ionization</b>	Soft	Soft (harsher than MALDI)
<b>Mass range</b>	For high mass, up to 500,000 Da	For small molecules, up to 200,000 Da
<b>Common mass analyser</b>	Commonly coupled to TOF analyser	Commonly coupled to quadrupole analysers
<b>LC-coupling</b>	Not compatible	Compatible
<b>Pros</b>	<ul style="list-style-type: none"> <li>- Very easy to operate</li> <li>- able to analyze proteins down to femtomole quantities</li> <li>- suitable for in-gel digestion approach</li> <li>- can tolerate small amounts of contaminants</li> <li>- more reliable in non-redundant peptides ionisation</li> <li>- highly reproducible</li> </ul>	<ul style="list-style-type: none"> <li>- highly efficient and automated sample throughput</li> <li>- continue ions flow</li> <li>- More efficient in fragmentation (works in acidic pH)</li> <li>- Multiple proton-accepting sites generated</li> </ul>
<b>Cons</b>	<ul style="list-style-type: none"> <li>- Discontinue ions flow</li> <li>- Greatly affected by the quality of the sample, which can be contaminated.</li> <li>- Time consuming</li> <li>- Matrix produces background less than 800-900 m/z, problematic for small molecules analysis</li> </ul>	<ul style="list-style-type: none"> <li>- Multiple fragmentations, complicates downstream analysis</li> <li>- Less suitable for non-basic, low-polarity molecules</li> <li>- Ion suppression due to high concentration of non-volatile compounds</li> </ul>

One of the main differences between MALDI and ESI is the generated fragments during MS/MS phase. MALDI produces more ions series of 1<sup>+</sup> fragments including ammonium, a, b, y, x, d, v, and w ions. On the other side, ESI generates 2<sup>+</sup> fragments or higher with only b and y ions (Figure 1.12), which might indicate more peptide sequence coverage by MALDI.



**Figure 1-12. Mass spectrum for a common peptide of CYP1a2 (IGSTPVVLSGLNTIK) from mouse liver microsomes analysed in the current project using MALDI-TOF-TOF-MS MS/MS (A) and previous project using ESI-Orbitrap Fusion MS-MS/MS (B).**

The matrix plays a key role in MALDI-MS by; (i) absorbing the laser energy and heating up the matrix to create a gas plume, (ii) being in excess, it protects the analytes from highly energy-laser source by absorbing most of the incident energy, (iii) separating the analyte molecules and thereby preventing the

formation of sample clusters that inhibit the appearance of molecular ions and (iv) serving as a proton donor and receptor, which is important for ionizing the analyte in both positive and negative ionization modes, respectively (Lewis et al., 2006, de Hoffmann and Stroobant, 2007). Such examples of compounds most frequently used as a matrix are  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) for peptides below 5 kDa, 2,5- dihydroxybenzoic acid (gentisic acid) for carbohydrates and *trans*-3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) used for protein analysis (de Hoffmann and Stroobant, 2007).

#### **1.5.4.2 Mass analyser**

The mass analyser is the part where gaseous ion separation takes place based on their mass-to-charge ( $m/z$ ) ratio. Different types of mass analyser have developed, which vary in their physical principles and analytical performance capabilities including: mass range, analysis speed, resolution, sensitivity, ion transmission, and dynamic range (Seitz and Schumacher, 2015). There are four different types of mass analysers which are commonly used for proteomics research: quadrupoles, ion trap (IT), time of flight (TOF), and Orbitraps (Balch and Yates, 2011). As an MALDI source produces short pulses of ions, it is preferably coupled to a very fast mass analyser such as TOF analyser, which measures the time taken by gas phase-ions to travel from ionization source to detector ( $m/z$ ) (Yates et al., 2009). In contrast, ESI source produces a continuous current of ions; making it suitable to be coupled to a quadrupole, ion trap, and/or Orbitrap mass analyser. Since different mass analysers have strengths and weaknesses in their performance, which are characterized by the resolution, accuracy, sensitivity, mass range of detection

and cost, hybridizing two means of mass analyser for ionized analytes separation can provide greater experimental flexibility and reliability (Table 1.6) (De Carolis et al., 2014, Domon and Aebersold, 2006). An example of such hybridization is combining LIT that has a low-resolution power but high sensitivity with Orbitrap which has relatively low sensitivity but excellent resolving power (Michalski et al., 2012). However, coupling mass analysers together is used primarily to perform tandem mass spectrometry (MS/MS) analysis, where specific peptides are isolated in the first analyser, fragmented by collision with a gas, before separation in the second mass analyser.

#### **1.5.4.3 Detector**

The detector converts an ion current into an electrical current, which is then digitized and delivered to the PC (de Hoffmann and Stroobant, 2007).

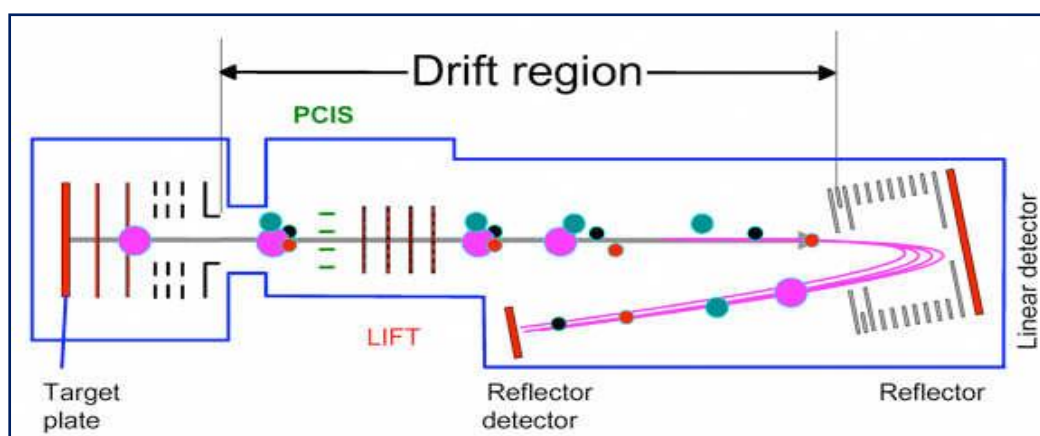


**Table 1-6. Performance comparison of selective mass analyzers, showing the pros and cons for each analyzer. The table is adapted from (Yates et al., 2009, Lemière, 2001, Kromidas, 2016).**

Mass analyser	Example	Resolution	Mass Accuracy	Sensitivity	Dynamic Range	Advantages	Disadvantages
<b>Scanning</b>	Time of flight (TOF)	10,000	2-5 ppm	Femtomole	$1 \times 10^6$	<ul style="list-style-type: none"> <li>• Good resolution and mass accuracy</li> <li>• Excellent mass range (m/z)</li> <li>• Simplest mass analyser.</li> <li>• Compact and easy to manipulate instrument</li> <li>• Easily adapted to MALDI</li> <li>• Low cost</li> <li>• Moderate to fast scan speed</li> </ul>	<ul style="list-style-type: none"> <li>• Less adapted to ESI than MALDI.</li> <li>• Not quantitative</li> </ul>
<b>Ion beam</b>	Triple quadrupole	2000	100 ppm	Attomole	$3 \times 10^3$ - $4 \times 10^3$	<ul style="list-style-type: none"> <li>• Easily adapted to ESI</li> <li>• Good sensitivity</li> <li>• Low cost</li> <li>• Small size</li> <li>• Ease to switch between positive and negative ions</li> <li>• Fast scan speed</li> <li>• Quantitative</li> </ul>	<ul style="list-style-type: none"> <li>• Limited mass range</li> <li>• Relative low mass accuracy and resolution.</li> <li>• Poor adaptability to MALDI</li> </ul>
<b>Trapping</b>	<b>Ion trap (IT):</b> <ul style="list-style-type: none"> <li>• 3D ion trap (QIT)</li> <li>• Linear ion trap (LIT)</li> </ul>	2000	100 ppm	Femtomole	Up to $1 \times 10^6$	<ul style="list-style-type: none"> <li>• Reasonable resolution</li> <li>• High sensitivity</li> <li>• Fast scan rate</li> <li>• Low cost</li> <li>• Small size</li> <li>• Excellent mass range</li> </ul>	<ul style="list-style-type: none"> <li>• Limited resolution</li> <li>• Relatively low mass accuracy</li> </ul>
	Orbitrap	500,000	2 ppm	Attomole	$3 \times 10^3$ - $4 \times 10^3$	<ul style="list-style-type: none"> <li>• Easily adapted to MALDI and ESI</li> <li>• Excellent resolution</li> <li>• Ease to switch between positive and negative ions</li> <li>• Low cost</li> <li>• Small size</li> <li>• Very fast scan speed</li> </ul>	<ul style="list-style-type: none"> <li>• Limited mass range</li> <li>• Relative low mass accuracy and sensitivity</li> </ul>
	Fourier transform-ion cyclotron resonance (FT-ICR)	500,000	<2 ppm	Femtomole	Up to $1 \times 10^4$	<ul style="list-style-type: none"> <li>• Good mass range, up to 10,000 m/z</li> <li>• Excellent mass accuracy and resolution</li> <li>• Easily adapted to MALDI and ESI</li> <li>• Well suited to analyse complex mixtures</li> <li>• Slow scan speed</li> </ul>	<ul style="list-style-type: none"> <li>• Expensive, require superconducting magnet</li> </ul>

#### 1.5.4.4 MALDI-TOF MS

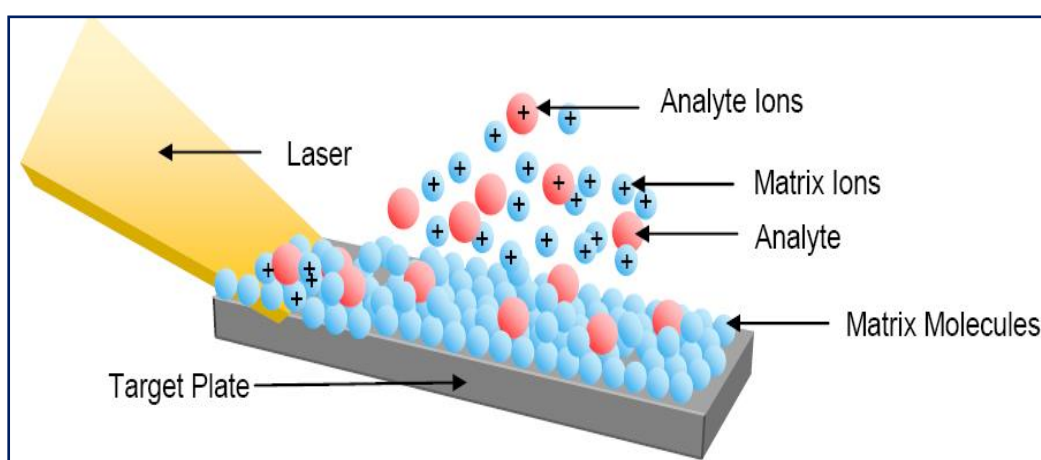
Proteomics Facility at the University of Bradford carried out their research using MALDI-TOF-TOF-MS, (Ultraflex II MALDI-TOF/TOF, Bruker Daltonics, Bremen, Germany). Schematically MALDI-TOF MS is composed of three main parts: source, where ions are produced and accelerated to a constant kinetic energy by means of electric fields; a drift region, which is a field-free region with a bounded extraction grid at ground potential; and a detector, where ions are colliding at the end, with the whole system under a high vacuum (Figure 1.13).



**Figure 1-13.** The schematic of Bruker Daltonics Ultraflex II MS consisted of target plate chamber, ion drift region and detectors. The target plate is introduced first into the ion source section at stage 1 (IS1) under vacuum. The laser beam hits the plate at the ground potential stage (P1). In the drift region, the precursor ion selector (PCIS) module is in charge to select for particular ion masses, which is then followed by the LIFT module to increase the kinetic energy of parent and fragment ions for detection. For better fragment ion detection, the parent ion signals can be deflected by using the post-LIFT metastable suppressor (PLMS) module. The signals are either measured in linear mode (MS mode) or reflected and detected by the reflector detector (de Hoffmann and Stroobant, 2007).

#### 1.5.4.5 Instrumentation

The first step involves the formation of co-crystals by mixing samples with excess matrix and loading it as spots on a metal plate. Upon complete evaporation of the sample's solvents, the plate is placed in the source where the dried spot is fired by laser pulse beam, generating a short burst of ions in the gaseous phase as shown in Figure 1.14.

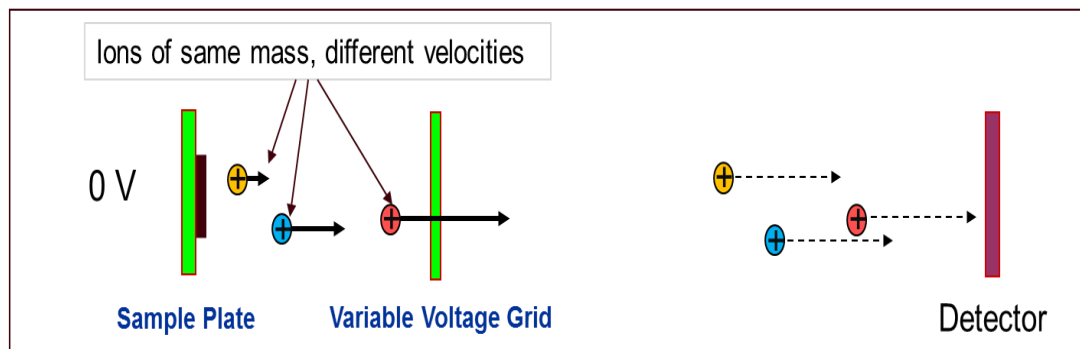


**Figure 1-14. Ionisation of analytes by MALDI. Sample co-crystallized first with the matrix. Then sample spot is irradiated by intense pulse laser beam, leading to sublimation and ionization of peptides. On the Ultraflex II MALDI-TOF MS, the sample is loaded onto a microtiter plate format MALDI target (metal plate for 384 sample spots) (de Hoffmann and Stroobant, 2007).**

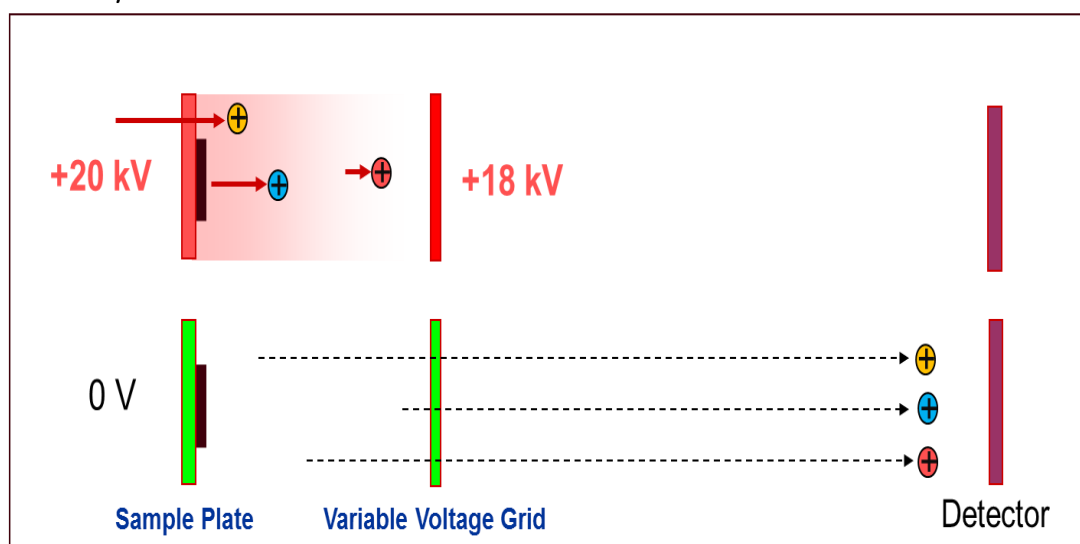
Generated ions are then separated using time of flight (TOF) mass analyser. However, since not all ions are absorbed and ionized at the same time and place, ions of the same mass do not have the same kinetic energy after passing the acceleration field. Therefore, about 100-500 ns after the laser pulse, a strong acceleration field is switched on (delayed extraction) (GmbH, 2006). Delayed extraction aims to impart a fixed kinetic energy to the ions

produced by MALDI process, ensuring that ions are accelerated and enter the drift region at the same time as illustrated in Figure 1.15.

#### A: Continuous Extraction



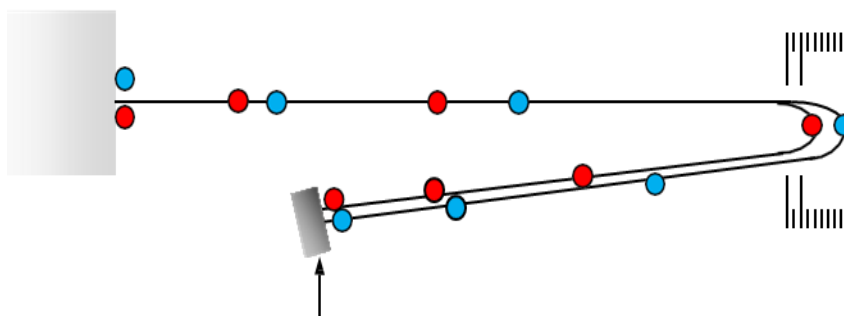
#### B: Delayed Extraction



**Figure 1-15. Schematic illustration of continuous (A) and delayed (B) extraction mode in a TOF. In continuous extraction mode, the electrical field is off, where; yellow, blue and red ions have the same mass with different kinetic energies (velocities) and so faster ion (red) hit before the slower one the detector. In delayed extraction mode, the electrical field is applied and a potential gradient accelerates slow ions more than fast ions resulting in those slow ions catch up with faster ones at the detector. Adapted from (Kovtoun et al., 2002).**

Throughout the flight tube, ions are reflected in a mirror called reflectron. It is basically an electrostatic reflector which reflects ions back through the flight tube till they reach the detector. The reflectron allows highly kinetic energy

ions to move more deeply into the reflector than ions with a lower energy (Figure 1.16). This compensates any differences in the initial energy and allows separating the ions with high resolutions on the basis of their mass-to-charge ratios.



**Figure 1-16. Schematic of a reflectron; ions (red and blue) have the same mass but not the same kinetic energies. Blue ion has much higher of kinetic energy compared to the red one, therefore it's travel more deeply. However, after being reflected by the reflector, both ions will have the same kinetic energy and hence hit the detector at the same time (de Hoffmann and Stroobant, 2007).**

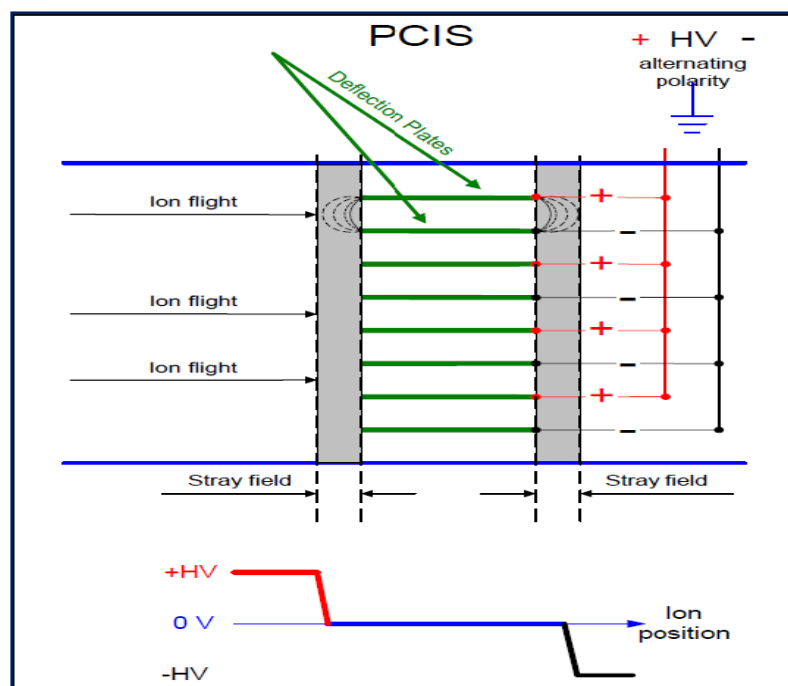
#### **1.5.4.6 Tandem MS**

Tandem mass spectrometry (MS/MS) is a combination of multiple stages of mass analyser, in tandem, and in the same mass spectrometer. This can be useful in the detection of both parent and fragment ions and consequently identification of peptide sequences. In contrast to MS/MS, MS mode is able to detect only parent ions and not the fragmented ones.

Bruker Daltonics Ultraflex II is an MALDI-TOF/TOF tandem mass spectrometer which, has two mass analysers making it an optimal technique for robotic MS and MS/MS throughout the identification of peptides and proteins (Figure 1.13). In MS/MS analysis, selected parent ions disintegrate

into fragments by means of collisionally induced dissociation (CID), and the fragments are analysed by TOF mass analyser. During the MS analysis, the feature of LIFT module is disabled and therefore only parent ions can be analysed and detected. When the LIFT device is used in MS/MS the kinetic energy of the selected parent and fragment ions is raised, allowing detection of fragments and parent ions. The LIFT module results in different kinetic energy for parent and fragment ions, where the level of difference between parent and smallest fragment does not exceed 30%. This difference allows for both parent and fragment ions to travel down the flight tube with the same velocity. Upon entering the reflectron the fragment ions have lower kinetic energy, so they do not travel as deep as parent ions do and are detected by the reflectron detector in a shorter flight time than the parent ions (GmbH, 2006).

The precursor ion selector (PCIS) is a mass filter that selects particular mass of parent ions and their related fragments to travel down a flight tube and then separates the selected ions from others for MS/MS analysis (Figure 1.17). It consists of deflector plates arranged in vertical layers below each other. Consecutive electrodes are coupled to a high voltage supply with alternating polarity. PCIS selects particular parent and fragment ion masses by altering the electrical fields during their entry into the deflector region to prevent deflection and allow ions to continue through the drift region (de Hoffmann and Stroobant, 2007).



**Figure 1-17. A schematic of precursor ion selector (PCIS) and high voltage alteration.** In the deflection region, the electrical field between the deflector layers is re-adjusted with aims to select particular parent and fragment ion masses. Thus, helps to prevent the deflection and allows them to move down the drift region (de Hoffmann and Stroobant, 2007).

The detector converts an ion current into an electrical current, which is then digitized and delivered to the PC. Because small ions have a higher velocity than large ions, the detector detects and records the small ions earlier than large ions, producing the TOF spectrum.

#### 1.5.4.7 Peptide identifications

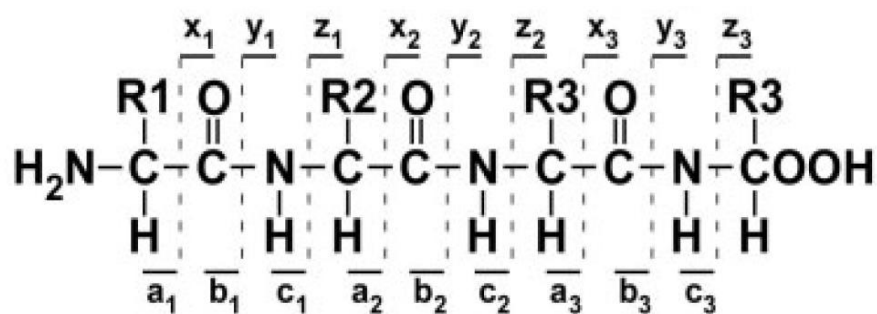
Protein identification can be achieved by identifying the sequences of the peptides. Proteins are first digested enzymatically using a restriction enzyme such as; trypsin, into shorter length peptides. Each peptide produced from protein is made of a characteristic sequence of amino acids and thereby it has its particular mass and the detection of a number of peptides from a digested

protein constitutes a unique pattern; this is known as “Peptide Mass Fingerprint” (PMF) (Pappin et al., 1993b, Henzel et al., 2003). The PMF list is then compared, using a database search engine, with theoretical peptide molecular masses (*in-silico* database) generated using the same restriction enzyme to create a list of best matches (Liska and Shevchenko, 2003).

The best-matched protein is most likely to be the correct protein, however, particular parameters such as scoring the quality of the match between the experimental and theoretical are used to validate the identified proteins by this method. Although PMF is a straightforward method that has been used widely in single purified protein identification, it is not feasible if the sample is a mixture of proteins or if there is an amino acid substitution or post-translational modifications (Liska and Shevchenko, 2003).

The MS/MS approach can overcome these limitations. Fragmenting the peptide using CID breaks the peptide backbone during the fragmentation process. The resultant amino-acid specific daughter ions provide information about the constituent amino-acids of the peptide, which is used to identify the peptide. The cleavage of the peptide bond is based on the physio-chemical properties of amino acids within the peptide (Hubbard, 2010). After cleavage, the charge can be retained on daughter fragments at a different location. If the charge is possessed upon the *N*-terminus, then ions are known as, “a”, “b”, “c” will be formed; on the contrary, “x”, “y”, “z” is formed if the charge is possessed upon the *C*-terminus (Figure 1.18). However, b and y fragment ions are the most common formed for CID cleavage.





**Figure 1-18. General product ion fragments generated upon fragmentation of from a peptide sequence in the mass spectrometer. The products (a, b, c) represent the N-terminus, where (x, y, z) represent the C-terminus after the fragmentation. Collision induced dissociation (CID) fragmentation generates mostly b and y ion series adapted from (Hubbard, 2010).**

The identification of peptides forms MS/MS analysis can be achieved by matching the MS/MS spectra from experimentally trypsin-digested peptides to the theoretically determined fingerprints, again using database searching. Imported mass values are scored in a way that allows peptides or proteins which best matches the data to be identified. Several algorithms and computer programmes have been designed to search protein sequence databases and identify proteins (e.g., SEQUEST, Mascot, Comet, etc.). ‘Mascot’ is a search engine, which employs probability in proteins identification. The mascot is a development of the molecular weight search engine (MOWSE), which was developed by Darryl Pappin and Alan Bleasby in 1993 (Pappin et al., 1993a) for peptide mass fingerprint (PMF). The mascot can conduct three different types of searches, Peptide Mass Fingerprint; Sequence Queries; and MS/MS ion searches [<http://www.matrixscience.com/>]. Probability-based scoring is applied to search engines in order to establish the confidence of matching an ‘unknown’ protein to the closest sequence homology on the database and

determine if it is significant or not, in order to reduce false positive results. In this approach, the probability of a match between the experimental data and theoretical mass values is calculated from candidate protein or protein sequence compared to a random event. A very low probability value is reported as the real match. Because the best match is usually a very small number, the Mascot score has been used instead of probability (P) which is equal to  $(-10 \log_{10} (P))$ . Hence, the higher the MASCOT score, the more likely the peptide derives from the identified protein. In addition, the significance of the real match depends also on the size of the database.

## **1.6 Aims and objectives**

The objective of the project is to investigate the influence of major anti-cancer drugs on mouse liver protein expression, in particular, the microsomal fraction, since the liver is considered as the primary organ in the body responsible for drugs metabolism and detoxification, and it has been involved in several drugs toxicity and safety profile studies.

The expected outcomes of this work are: (i) demonstrating the optimised quantitative proteomics workflow to be used henceforth in liver microsomes investigation, (ii) identifying and validating potential biomarkers from treated liver microsomes in response to major anti-cancer drugs, (iii) better understanding of the influence of these drugs on the expression and activity of chief enzyme family exist mainly in liver microsomes (i.e. CYP450 subfamily enzymes) and, (iv) assessing the influence of sex variation affecting the expression and activity of pre-defined proteins. The approaches taken to accomplish these goals are outlined and the structure of this thesis is as

follows; the introductory chapter (Chapter 1) provides the background and motivation for the research, the research objectives and an overview of the research approach. Prior to this research, Chapter 2, describes the materials and methods common to much of the work contained within this thesis, more specific methods can be found within the chapter to which they pertain. Chapter 3 describes the optimisation of protein extraction by four different extraction methods followed by two key peptides-separation techniques. Hence, Chapter 4 investigates the impact of 5-flourouracil, doxorubicin, paclitaxel, cisplatin, and the CYP450s inducer, 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) on liver microsomes proteomes from mice. Chapters 3 and 4 aims were accomplished using quantitative proteomics approach (iTRAQ labelled), nano-reverse phase HPLC and MALDI-TOF-MS MS/MS. Identified proteins from Chapter 4 were validated in a new highly controlled study involved both sexes in Chapter 5, with definitive goals to confirm the regulated proteins in shotgun study (Chapter 4) using the advances of Western blotting technique and exploring the influence of sex variation in response to two anti-cancer drugs (Paclitaxel and Doxorubicin). Chapter 6 correlates the enzyme activity of two CYP450 isoforms (CYP1A2 and CYP3A4) in response to drugs treatment using fluorescence assay (VIVID assay) with their expression level as indicated by Western blotting analysis in Chapter 5. The seventh and final chapter of the thesis gives a summary of the conclusions drawn from the presented research, and recommendations for future works.

## Chapter two: General materials and method

## **2 General materials and method**

### **2.1 Materials**

#### **2.1.1 Proteomics analysis**

The highest quality reagents were used throughout, from the following sources;  $\alpha$ -cyano-4-hydroxy-cinnamic acid (CHCA), Peptide Calibration Standard II (components: angiotensin I, angiotensin II, substance P, bombesin, ACTH clip 1-17, ACTH clip 18-39, somatostatin 28, bradykinin fragment 1-7 and renin substrate tetradecapeptide porcine; covered mass range ~700Da – 3200Da.) from Bruker Daltonics GmbH, Bremen, Germany; HPLC grade water, HPLC grade acetonitrile (ACN), HPLC grade methanol (MeOH), HPLC grade ethanol from Fisher Scientific, Leicestershire, England; trypsin (modified sequencing grade), complete mini EDTA-free protease inhibitor cocktail tablets (PIC) from Roche Diagnostics GmbH, Berlin, Germany; urea, sodium dodecyl sulphate (SDS), ammonium bicarbonate, phosphate buffered saline (PBS), acetone, iodoacetamide (IAA), dithiothreitol (DTT), trifluoroacetic acid (TFA), Bradford reagent, albumin from bovine serum (BSA), ammonium phosphate monobasic, potassium chloride (KCl), hydrochloric acid (HCl), sodium azide (NaN<sub>3</sub>), triethylammonium bicarbonate (TEAB) from Sigma-Aldrich, Poole, UK; potassium dihydrogen phosphate (Analar, Belgium); iTRAQ 4-plex reagent (AB Sciex UK Limited, Warrington, UK). OFF-GEL Kit (thiourea, dithiothreitol, glycerol and OFF-GEL buffer), Rehydration Solution and Mineral oil, pH 3-10, were from Agilent Technologies, CA, USA.

### **2.1.2 SDS-PAGE electrophoresis and Western blotting**

Acrylamide (30%) and tris-hydroxymethyl aminomethane (Tris) were purchased from Bio-Rad laboratories, Hercules CA, USA; Mouse anti-CYP1A2 (ab22717), MUP1 recombinant protein (ab95193) and Rabbit anti-CYP2E1 (ab151544), anti-CYP3A4 (ab135813), were purchased from Abcam, Cambridge, UK; Mouse anti- $\beta$  actin (A2228), anti-mouse IgG (A4416), Coomassie Brilliant Blue stain, glycerol, glycine, sodium chloride, tetramethylethylenediamine (TEMED), Tween-20, 'PhastGel Blue R' Coomassie Brilliant Blue tablets and  $\beta$ -mercaptoethanol were purchased from Sigma-Aldrich, Poole, UK; Rabbit anti-MUP1 antibody was purchased from Santa Cruz, Texas, USA; Anti-Rabbit IgG-conjugated with HRP was purchased from Dako, Glostrup, Denmark; HPLC grade water, methanol and isobutanol and PageRuler™ Plus Prestained Protein Ladder (10 to 250 kDa) were purchased from Fisher Scientific UK Ltd, England; Amersham ECL-plus, Amersham hyperfilm™ ECL and ammonium persulphate (APS) were purchased from GE Healthcare, Buckinghamshire, UK; Multi-grade Rapid developer and fixer were purchased from ILFORD Imaging, Cheshire, UK; Skimmed milk from Marvel Premier Food, Manchester, UK.

### **2.1.3 Enzyme activity assay**

VIVID CYP450 Reaction Buffer I, VIVID Assay Substrate, 0.1 mg of (7-benzyloxy-methyloxy-3-cyanocoumarin, BOMCC for CYP3A4 or substrate 7-ethoxymethoxy-3-cyanocoumarin, EOMCC for CYP1A2), baculosomes CYP3A4 (0.5 nmol) and CYP1A2 (0.5 nmol), 10mM Vivid NADP<sup>+</sup>, and VIVID regeneration system were purchased from Life Technologies, Carlsbad, CA.

## **2.2 Methods**

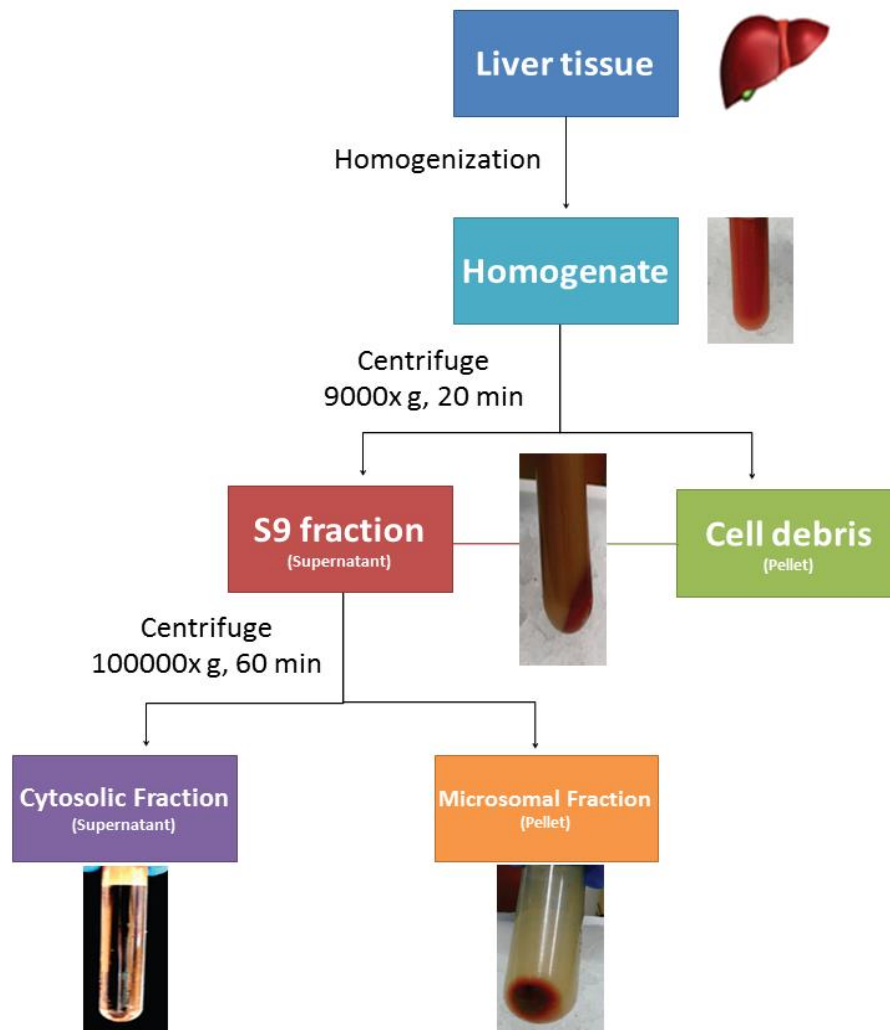
### **2.2.1 Proteomics analysis**

#### **2.2.1.1 Tissue homogenization and protein extraction**

As a part of extraction protein optimization, four different mechanical extraction methods were evaluated and described in Chapter 3, section 3.2.2.1.

#### **2.2.1.2 Microsomal fraction preparation**

Liver microsomes were prepared from S9 fraction of liver extracts using a differential centrifugation method (Figure 2.1) as described previously (Sutton et al., 2010). Briefly, the supernatant (S9) was transferred to ultracentrifuge tubes and centrifuged at 100,000 *g* using the Beckman Optima TL100 Ultracentrifuge (Beckman Coulter, UK) for 60 minutes at 4°C to sediment microsomes. The cytosolic supernatant was transferred to fresh tubes and stored at -80°C for future use. The microsomal pellet was then resuspended with 0.5 ml ammonium bicarbonate for protein concentration determination.



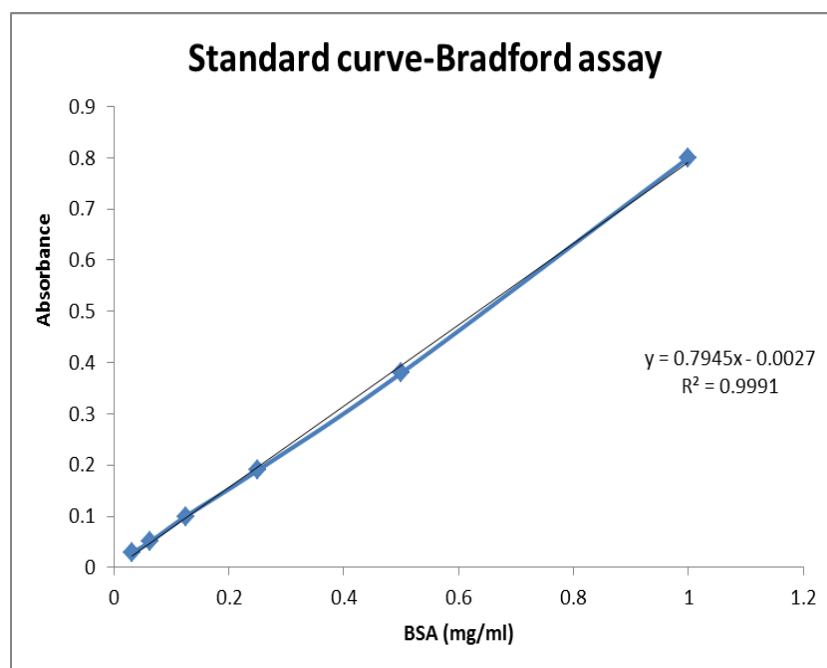
**Figure 2-1. Flow diagram for Ultracentrifugation method**

### **2.2.1.3 Protein Assay**

Bradford assay was used to determine the initial protein concentrations in solution and ensure that the same amount of protein was used in further experiments (Bradford, 1976). A standard curve of absorbance versus serial concentrations of bovine serum albumin (BSA) was created starting from 1mg/ml to 0.0625mg/ml (Figure 2.2). Absorbances were detected using a Multiskan Spectrum spectrophotometer (Thermo Scientific, Vantaa, Finland) at a wavelength of 595nm by adding 1.5 ml of Bradford reagent to 50 µl of



standards, and then standards were incubated for 15 minutes at room temperature. The samples were prepared in the same way, analysed in duplicate and the readings extrapolated to the standard curve to calculate samples concentrations.



**Figure 2-2. BSA standard curve represents the absorbance of serial dilutions of BSA.**

#### **2.2.1.4 Protein Desalting and Concentration**

Briefly, pre-cooled acetone (1 ml) was added to a 10-fold excess of a sample containing approximately 50 $\mu$ g of extracted protein and the mixture stored at 20°C overnight. Thereafter the mixture was centrifuged for 20 minutes, 13,000 *g* at 4°C. The supernatant was discarded and the pellet was lyophilized and kept on ice for further analysis.

#### **2.2.1.5 Protein digestion**

In order to convert proteins into peptides that can be used for tagging and identification, lyophilised protein pellets (equivalent to 50µg) were resuspended in 5 µl of 8M urea, 400mM ammonium bicarbonate. The resuspended pellet was reduced by 1 µl of 50mM dithiothreitol (DTT) and incubated in a water bath at 80°C for 20 minutes. The sample was cooled to room temperature and alkylated with 1 µl of 100mM iodoacetamide (IAA) for 20 minutes in the dark. After that, 13 µl of 400mM ammonium bicarbonate and 10% acetonitrile (ACN) were added to reduce the concentration of urea to 2M, before 2 µl of 1mg/ml trypsin was added and incubated at 37°C for 18-20 hours.

#### **2.2.1.6 Digested proteins purification and desalting**

The digested samples were desalted using TELOS® C18 disposable columns (Kinesis Ltd., Cambridgeshire, England) to remove urea and other modifying reagents. Initially, the columns were wet with 1 ml of 100% methanol and then equilibrated with 2 ml of solvent A (2% v/v ACN and 0.05% TFA). A mixture of the digested sample (100 - 500 µl) and solvent A (2% v/v ACN and 0.05% TFA) was added to the column, with the total volume adjusted to 500 µl. The column was then washed with 2 ml of solvent A (2% v/v ACN and 0.05% TFA). The bound proteins were eluted using 1 ml of solvent B (80% v/v ACN and 0.05% TFA) and collected in an Eppendorf tube. Finally, samples were centrifuged and lyophilized at 45°C to dryness for storage at -20°C.

#### **2.2.1.7 iTRAQ peptide labelling**

Ethanol (70 µl) was used for iTRAQ 4-plex reagent resuspension, each vial contents were transferred to the relevant protein digested tubes. iTRAQ vials were rewashed with 10 µl ethanol, vortexed, then transferred to relevant sample tubes, vortexed and centrifuged at 14,100 *g*. The pH was tested and adjusted to pH 8.0 using 1M TEAB if required. After 2 hours of incubation at room temperature, the pH was retested and adjusted if needed. The contents of all the tubes were combined into one tube after adding 50 µl HPLC-water to stop the reaction. A further 25 µl of HPLC water was added to each tube, to wash out the sample, and combined with the remainder. The combined samples were lyophilized at 45°C and then stored at 4°C for further analysis.

#### **2.2.1.8 Peptides separation**

After protein digestion and labelling, peptides were separated in two dimensions, based on charge and then hydrophobic properties, to extend the coverage of identified proteins. However, for the first dimension separation; two charge-based techniques (strong cation exchange and isoelectric-focusing) were compared to find the best resolving technique (Chapter 3).

##### **2.2.1.8.1 First-dimensional separation**

###### **2.2.1.8.1.1 Strong cation Exchange**

The combined iTRAQ-labelled peptides were separated using ISOLUTE® column (ITS Ltd., England). Loading buffer (200 ml) was prepared using 10mM potassium dihydrogen phosphate in 25% (v/v) ACN and 0.01% (w/v) sodium azide (NaN<sub>3</sub>), and then adjusted at pH 3.0. The loading buffer was used to wet

the column and to resuspend the sample by using 2 ml and 0.6 ml, respectively. The resuspended sample was loaded onto the equilibrated column from which the first fraction (flow through 1) was collected by passive hydrostatic pressure. Then, the column was washed with 1 ml of loading buffer and collected as flow through-2 fraction. In order to elute the bounded peptides, 500 µl of a serial concentration of potassium chloride (30mM to 1000mM) in loading buffer were applied and the resulting eluate collected as shown in Table 2.1. A total of 12 fractions were collected for second dimension separation of peptides.

**Table 2-1. Serial concentrations of potassium chloride used to elute bounded peptides.**

<b>Fraction</b>	<b>Volume loading buffer</b>	<b>Concentration of KCl (mM)</b>	<b>Amount of KCl for 10ml (mg)</b>
<b>E1</b>	10 ml	30.0	22.4
<b>E2</b>	10 ml	60.0	44.7
<b>E3</b>	10 ml	90.0	67.1
<b>E4</b>	10 ml	120.0	89.5
<b>E5</b>	10 ml	150.0	111.8
<b>E6</b>	10 ml	180.0	134.2
<b>E7</b>	10 ml	250.0	186.4
<b>E8</b>	10 ml	300.0	223.7
<b>E9</b>	10 ml	350.0	260.9
<b>E10</b>	10 ml	500.0	372.8
<b>E11</b>	10 ml	700.0	521.9
<b>E12</b>	10 ml	1000.0	745.5

Indeed, the eluted samples (fractions) were desalted as described before in (section 2.2.1.6), lyophilized at 45°C and kept at -20°C.

#### **2.2.1.8.1.2 Isoelectric-focusing**

Isoelectric-focusing (IEF) separation for iTRAQ labelled peptides was performed using the Agilent 3100 OFF-GEL Fractionator (Agilent Technologies) according to protein or peptide isoelectric point (pI), whereby the separated component was recovered in liquid fractions.

Samples were resuspended in peptide OFF-GEL stock solution 1.25X. Immobilized pH gradient (IPG) strip gel 12 or 17 cm (Agilent Technologies) was placed on the tray well and the tray frame placed over the gel. The gel was rehydrated with 40 µl of IPG strip Rehydration solution per well and then 150 µl of resuspended sample was loaded into each well. Mineral oil was used to cover the gel strip ends, after that the electrodes were fixed at both ends of the gel strips. Peptides were focused for 20 kV for 24 hours and the results were either 12 or 24 fractions based on the length of gel strip (12 or 17 cm). Fractions were collected and desalted in accordance with the procedure described in section 2.2.1.6. The lyophilized samples were kept at -20°C for the second dimension separation.

#### **2.2.1.8.2 Second-dimensional separation**

Throughout this project, the second dimension separation of peptides was carried out using a Nano-scale Reverse phase liquid Chromatography, LC Packings UltiMate 3000 capillary high-performance liquid chromatography system (Dionex, Surrey, UK). Utilising a PepMap100 C18 5µm, 5 cm guard column and a PepMap 100 C18 3µm, 15 cm analytical column (LC Packings,

Sunnyvale, USA). Lyophilised iTRAQ-labelled samples were resuspended in 13 µl of 10% (v/v) ACN and 5 µl was injected onto the precolumn through the auto-sampler system. The sample was washed with mobile phase A, (2% v/v ACN, 0.05% v/v TFA) for 3.5 minutes at a flow rate of 0.3 µl/min on the pre-column before being switched onto the analytical column. Six minutes after sample injection, the mobile phase B (80% v/v ACN, 0.05% v/v TFA) was increased to 15% and then increased with a linear gradient to 45% over 105 minutes. At minutes 111.1 the mobile phase B was increased from 45% to 100%. This mobile phase composition is maintained for 5 minutes and then changed to 100% mobile phase A ready for the next sample. During the analytical phase (16 to 112 minutes), a total of 384 fractions, at 75 nL for each, were collected onto an MTP Anchor Chip 800/384 target plate (Bruker Daltonics) using a Proteiner FC fraction collector (Bruker Daltonics). As each fraction was collected, 1.5 µl of a saturated α-cyano-4-hydroxycinnamic acid (CHCA) working matrix solution (1.056 ml ethanol: acetone, 120 µl saturated CHCA in 30% v/v ACN, 12 µl 100mM ammonium phosphate, 12 µl 45% v/v TFA) was automatically co-deposited by the fraction collector. The resultant droplets were allowed to air-dry and Peptide Calibration Standard II (Bruker Daltonics) was added manually using 0.4 µl Peptide II calibrant standards and 1.5 µl CHCA working matrix solutions between each group of four fractions.

#### **2.2.1.9 MALDI - TOF/TOF MS analysis**

Mass spectrometric analysis of protein sample was performed using an Ultraflex II MALDI-TOF/TOF mass spectrometer (Bruker Daltonics). Prepared samples on the MTP AnchorChip 800/384 target plate were loaded into the

mass spectrometer (MS). A fully automated run was carried out using WarpLC software package version 1.3 (Bruker Daltonics). The laser power for calibrant, sample, and target teaching were manually adjusted and saved in relevant executable methods using FlexControl software version 3.4 (Bruker Daltonics) to be included in the automatic run. The MS data acquisition of each fraction was performed and parent peak signals identified between 700 and 4300 Daltons using FlexControl-MS mode. WarpLC software creates a non-redundant list of masses with signal-to-noise ratio  $\geq 7:1$ , which are the subject of MS/MS analysis using FlexControl-LIFT mode. Mass peak lists were created for each compound which was transferred to ProteinScape software version 3.0 (Bruker Daltonics) for database searching against a SwissProt 52.4 mouse protein database containing 16,765 sequences. The search standard parameters were set up in ProteinScape as follows; Mascot search engine, taxonomy *Mus musculus* species, trypsin digestion, fixed modifications (carbamidomethyl of cysteine residues, iTRAQ modification of lysine residues and iTRAQ modification of N-terminal residues), variable modification (oxidation of methionine residues), 2 partial digests allowed and 100 parts per million (ppm) peptide mass tolerance for MS and 0.7 Daltons for MS/MS. For peptides acceptance, parameters were set for peptides as threshold score of 5, but proteins were only accepted if identified by at least one peptide with a score higher than 28 (a confidence level of 95% interval ( $p < 0.05$ )). Only the ranked first peptides were accepted.

#### **2.2.1.10 Data analysis**

ProteinScape Software version 3.0 (Bruker Daltonics) was used to generate a non-redundant protein list by combining all the fractions according to the previously mentioned parameters (section 2.2.1.9). The combined protein list was reprocessed manually, in which each single protein was investigated for a number of peptides; include replicate spectra for the same peptides, their Mascot scores and the inclusion of the only 1st ranked peptides.

Proteins identified were deemed significant if they were identified by at least two peptides or multiple peptide spectrum matches (PSMs) and the Mascot score was greater than 28 for at least one of these peptides. However, for proteins with high degree of sequence homology, such as CYP450 members, proteins were considered significantly identified if two peptides minimum were detected; at least one of them was unique and has a Mascot score greater than 28 or one unique peptide with at least two spectra; one of them with Mascot score greater than 28.

#### **2.2.1.11 Protein quantifications**

For protein quantification, the unique peptide ratios were used relying on the relative intensities of the reporter ions of the corresponding peptides. Unique peptide list was generated from peptide redundant list by excluding the common peptides and any other peptide that has less than two iTRAQ ratios. Moreover, at least two unique peptides were required for determining the protein relative amount or a single peptide with multiple peptide spectrum matches (PSMs) with coefficient variation percentage (CV%) not more than 25%. The p-value for each quantified protein was computed following the



permutation test as described by Nguyen and his colleagues (Nguyen et al., 2012). The p-value computations have been done automatically using the Quantitative Proteomics p-value Calculator (QPPC), which is available online for free at [<http://qppc.di.uq.edu.au/>]. Briefly, an excel file contained normalised unique peptide ratios was uploaded to the QPPC website. A standard parameter set was used, which includes the number of computation repetitions (number of permutations) as 10000 (Chen et al., 2014). The resultant is an excel sheet having a list of proteins with their mean peptide ratios.

For standard deviation determination and comparison between iTRAQ ratio datasets, data normalisation was performed for iTRAQ ratios by calculating the median protein value for each ratio (115/114, 116/114 and 117/114). Then, each protein value was divided by the average ratio to calculate the normalized ratio for each ratio (115/114, 116/114 and 117/114).

#### **2.2.1.12      Thresholds for significantly changed proteins**

Unique protein list was used for significant differentially expressed proteins were determined by applying two criterion as cutoffs for significantly altered proteins to reduce false positive. The first criterion was based on the normalised data as illustrated in Section 2.2.1.11. Briefly, the protein-normalised ratios were first converted into  $\log_2$  ratios and then standard deviation (SD) of  $\log_2$  ratios was calculated for each ratio. Finally, up-regulated or down-regulated proteins were defined as those with ratios greater than +1SD and those with ratios less than -1SD, respectively. The second cutoff was the p-value (calculated in previous section 2.2.1.10), while proteins with

95% confidence (p-value <0.05) were considered as significantly regulated protein (either up- or down-regulated) based on the values of iTRAQ ratios.

#### **2.2.1.13 Bioinformatics analysis of identified proteins**

Protein annotations were obtained primarily from the Protein Analysis through Evolutionary Relationships (PANTHER) software tool, which is available online at [<http://www.pantherdb.org>] (Stan et al., 2005). The protein analysis includes assigning the identified proteins to specific gene ontological definitions defined by cellular location, molecular function or biological process. Localization prediction of membrane proteins was determined based on the Gene Ontology (GO) information provided by UniProt database 7.0 [<http://www.uniprot.org/>] (Consortium, 2014). The grand average of hydropathy (GRAVY) score was used to evaluate the hydrophobic status of proteins, indicate a hydrophobic protein, and suggest a membrane association. GRAVY index was determined using the GRAVY calculator [<http://www.gravy-calculator.de/>]. A calculated GRAVY score of up to -0.4 indicates a hydrophobic protein (Kyte and Doolittle, 1982).

For integral membrane proteins prediction, a combined transmembrane topology and signal peptide predictor (Phobius tool) was used. It is an online tool provided by the European Bioinformatics Institute (EMBL-EBI) that can be accessed by submitting the sequence of amino acids for a specific protein in FASTA format [<http://www.ebi.ac.uk/Tools/pfa/phobius/>]. Phobius gives a list of the location of the predicted transmembrane helices, the predicted location of the intervening loop regions and signal peptide (Käll et al., 2004).

Skyline Targeted Proteomics Environment (Skyline 3.5-64 bit) software tool, was used to provide possible theoretical digests from SwissProt database of close homologues isoforms in order to confirm whether the particular peptide is unique for matched isoform (MacLean et al., 2010).

In additional, homology of closely related isoforms within subfamily was determined using multiple sequence alignment tools, CLUSTAL O (1.2.1), which is available on-line [<http://www.ebi.ac.uk/Tools/msa/clustalo/>] at the European Bioinformatics Institute (EMBL-EBI) website.

For enrichment/depletion test, the Cytoscape plugin, Biological Networks Gene Ontology (BinGO), was used to classify proteins according to their cellular component, molecular function, and biological process and then find statistically enriched and depleted GO categories of the protein dataset. A list of regulated protein was compared to a list of reference protein (International Protein Index – IPI of the mouse). Only significant proteins were considered either over-represented (enriched) or under-represented (depleted) by calculation of p-values. P-value was computed following the hypergeometric statistic test. This statistical test displays the probability that the number of genes observed in this category occurred by chance (randomly), as compared with the reference list (IPI). Categories were only considered significantly enriched when (i) p-values were < 0.01 after correcting for a multiple term testing with a Benjamini and Hochberg false discovery rate and (ii) categories included more than one protein.

## **2.2.2 SDS-PAGE electrophoresis and Western blotting**

### **2.2.2.1 Gel preparation**

Fresh SDS polyacrylamide gel was prepared manually by pouring the resolving gel (12% acrylamide, 1% SDS, 1% APS, 0.04% TEMED in Tris-HCl buffer) into the casting glass plates. Plates were attached to the casting frame on the casting stand (Bio-Rad). After that, the top layer of gel was temporarily filled with isobutanol. Once the gel had polymerized, after approximately 45 minutes, isobutanol was replaced by stacking gel (6% acrylamide, 1% SDS, 1% APS, 0.08% TEMED in Tris-HCl buffer). The 1 mm comb was immediately inserted to create the wells for sample loading and the gel left to polymerize for approximately 20 minutes. Gels were removed from the casting stand and frames and immediately utilised for SDS-PAGE.

### **2.2.2.2 Sample preparation**

A mixture of protein extracts (40µg) and 2X of SDS-reducing buffer (Laemmli buffer; 2% SDS, 25% glycerol, 5% β-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris-HCl, pH approximately 6.8) were heated to 65 °C for 15 minutes for protein denaturation.

### **2.2.2.3 Gel Electrophoresis**

The fresh gel prepared (section 2.2.2.1) was inserted into the Mini-PROTEAN electrophoresis system (Bio-Rad) that contains electrophoresis running buffer (25mM Tris, 192mM glycine, and 0.1%SDS). After assembling the system, the denatured protein samples (20µl of each) were loaded into gel wells. A lane was reserved for Prestained Protein Ladder separation named by marker lane.

Manual gel separation involved an initial power pack setting of 20 minutes at 80V followed by approximately 60 minutes at 150V (Bio-Rad). Then, the gel was used for either qualitative visual investigation by staining or for quantitative investigation by Western blotting technique.

#### **2.2.2.4 Coomassie Brilliant Blue staining**

After running the gel, the casting plates were disassembled, the gel removed and transferred into container containing distilled water to wash the gel from any excess SDS, after that, gel staining was done by immersing the gel in Coomassie Brilliant Blue stain solution (0.02% Coomassie Brilliant Blue R, 50% MeOH and 10% glacial acetic acid) for 45-60 minutes with gentle agitation. The stain was then replaced with destaining solution (50% MeOH, 5% acetic acid), which in turn was replenished several times with fresh destain solution until the background of the gel was fully removed. The destain solution was then replaced with distilled water to remove traces of stain and to rehydrate/expand the gel. Stained gels were stored in 10% MeOH at 4°C for further analysis.

#### **2.2.2.5 Western blotting**

As an alternative to Coomassie Blue staining, proteins separated in gels were transferred onto Hybond-P nitrocellulose membrane (GE Healthcare). A sandwich of the gel and nitrocellulose was immersed in a tank containing transfer buffer (48 mM Tris-base, 39 mM glycine, 0.04% SDS and 20% MeOH, pH8.3). Proteins were transferred on ice by electroblotting for two hours at 85 mA in a Mini Trans-Blot Cell (Bio-Rad). Blocking buffer-TBST (5% w/v skimmed milk dissolved in Tris-buffered saline, 0.05% v/v Tween-20) was then

incubated with the recovered membrane for one hour at room temperature to block the non-specific binding sites. Following that, the blot was incubated with an appropriate primary antibody (Table 2.2) in blocking buffer overnight (16 hours) at 4°C with constant agitation. The blot was then washed three times, 5 minutes each, with fresh TBST to remove the excess primary antibody before incubation with the appropriate secondary antibody in blocking buffer for one hour at room temperature (Table 2.3). Lastly, the blot was washed three times, 15 minutes for each with fresh TBST.

**Table 2-2. A table of different primary antibodies that have been used thoroughly in the present project. The table shows information about the antibody including, supplier, the source of antibody, reactivity and specificity, and optimized concentrations used. TBST; Tris-buffered saline plus 0.05% v/v Tween-20.**

Name of primary antibody	Species reactivity	Developed animal	Supplier	Dilution	Blocking buffer	Clonality	Epitope
Anti-Cytochrome P450 1A2 antibody	Mouse, Rat, Human	Mouse	Abcam (ab22717)	1/2500	TBST	monoclonal	Full length of CYP4501A2 (Rat)
Anti-Cytochrome P450 2E1 antibody	Mouse	Rabbit	Abcam (ab151544)	1/2000	TBST	monoclonal	Full length of CYP4502E1 (Rat)
Anti-Cytochrome P450 3A4 antibody	Human	Rabbit	Abcam (ab135813)	1/500	TBST	polyclonal	Synthetic peptide corresponding to human CYP3A4 (aa 234-264)
Anti-Major urinary protein 1 antibody	Mouse	Rabbit	Santa Cruz (sc-66976)	1/1000	TBST	polyclonal	Full-length protein of MUP1 (Mouse)
anti- $\beta$ actin antibody	Mouse, Rat, Human	Mouse	Sigma-Aldrich (A2228)	1/5000	TBST	monoclonal	N-terminal end of the $\beta$ -isoform of actin (Human)

**Table 2-3. A table of different secondary antibodies that have been used thoroughly in the present project. The table shows information about the antibody including, supplier, the source of antibody, reactivity and specificity, and optimized concentrations used.**

Name of primary antibody	Species reactivity	Developed animal	Supplier	Dilution	Clonality
Anti-mouse IgG conjugated with HRP	Mouse	Rabbit	Sigma-Aldrich (A4416)	1/20000	polyclonal
Anti-rabbit IgG conjugated with HRP	Rabbit	Goat	Dako (P0448)	1/5000	polyclonal

Protein bands were visualised by the enhanced chemiluminescence (ECL) system (GE Healthcare) and according to the manufacturer's guideline. Briefly, the blot was exposed to ECL reagents (A and B) for two minutes at RT before being enveloped in a hard plastic sheet. Then, in the dark room, the blot was exposed to hyper film ECL for a variable time ranging from 10 seconds to 30 minutes depending on the antibody. Hyperfilm was developed using Multi-grade rapid developer solution (ILFORD) for up to three minutes, followed by distilled water to remove the excess developer solution and at the end, the blot was fixed using the Rapid film fixer for up to five minutes.

### **2.2.1 Enzyme activity assay**

The VIVID CYP450 assay was conducted in accordance with the manufacturer's instructions. All reactions were performed in black flat-bottomed 96-well plates, Nunc™ (Thermo Fisher Scientific) with a final well volume of 100 µl. Concentrations were derived from the manufacturer's handbook before being optimized during experiments (Table 2.4).

**Table 2-4. Stock concentrations of main components of the VIVID assay.**

<b>Cofactor</b>	<b>3A4</b>	<b>1A2</b>
<b>CYP450 baculosomes</b>	1000 pmol/ml = 1 $\mu$ M	1000 pmol/ml = 1 $\mu$ M
<b>VIVID Substrate</b>	BOMCC, 2mM	EOMCC, 2mM
<b>NADP<sup>+</sup></b>	10mM	10mM
<b>G6P/G6PD</b>	333mM/30 U/ml	333mM/30 U/ml

NADP<sup>+</sup>; Nicotinamide adenine dinucleotide phosphate, G6P/G6PD; Glucose-6-phosphate /Glucose-6-phosphate dehydrogenase, BOMCC; 7-benzoyloxy-methyloxy-3-cyanocoumarin, EOMCC; 7-ethoxymethoxy-3-cyanocoumarin

For BACULOSOME VIVID assays, A master pre-mix (100X) was created using 4850  $\mu$ l of Reaction buffer I (200mM potassium phosphate buffer, pH 8.0), 100  $\mu$ l of Regeneration System (333mM Glucose-6-phosphate and 30 U/ml Glucose-6-phosphate dehydrogenase in 100mM potassium phosphate buffer, pH 8.0) and 50  $\mu$ l of either CYP3A4 or CYP1A2 baculosomes (baculovirus microsomes co-expressing human CYP450, NADPH-cytochrome P450 reductase and human cytochrome b5). A substrate mix (100X) comprised of 30  $\mu$ l NADP<sup>+</sup> and VIVID Assay Substrate, 25  $\mu$ l of 2mM BOMCC or 15  $\mu$ l of 2mM EOMCC for CYP3A4 and CYP1A2, respectively. The final volume for substrate mix was topped with reaction buffer I to 1000  $\mu$ l.

In order to measure the activity of CYP450, 90  $\mu$ l of master pre-mix was mixed with 10  $\mu$ l of the substrate mix in a well, the final concentrations were computed (Table 2.5). The plate was read using the Fluoroskan Ascent FL Microplate Fluorometer and Luminometer (Thermo Fisher Scientific, UK) continuously every minute for 60 minutes at 390 and 518nm emission and excitation wavelengths respectively.



**Table 2-5. Final concentrations for main components of VIVID assay per reaction.**

<b>Cofactor</b>	<b>3A4</b>	<b>1A2</b>
<b>CYP450 baculosomes</b>	5nM	5nM
<b>VIVID Substrate</b>	BOMCC, 10 $\mu$ M	EOMCC, 3 $\mu$ M
<b>NADP<sup>+</sup></b>	30 $\mu$ M	30 $\mu$ M
<b>G6P/G6PD</b>	3.33mM/0.3 U/ml	3.33mM/0.3 U/ml

NADP<sup>+</sup>; Nicotinamide adenine dinucleotide phosphate, G6P/G6PD; Glucose-6-phosphate /Glucose-6-phosphate dehydrogenase; BOMCC; 7-benzoyloxy-methyloxy-3-cyanocoumarin; EOMCC; 7-ethoxymethoxy-3-cyanocoumarin

For mouse liver microsomal activity, the VIVID assay was analogous to the baculosome VIVID assay. CYP1A2 or CYP3A4 was replaced initially with a serial dilution of microsomal proteins to determine the best concentration then the optimised one was used in further assays (protein content was determined as illustrated in section 2.2.1.3). The level of CYP450 activity was calculated by the change in relative fluorescent units (RFU) compared to a standard (Baculosome VIVID assays) with a known amount of CYP450 activity. All experiments were conducted in duplicate to confirm reproducibility. Statistical analysis was performed using GraphPad 7.0 software following either a Students' t-test or ANOVA. Data were presented as means  $\pm$  standard deviation (S.D.). Significance was noted at  $P < 0.05$ .

## Chapter three: Optimisation of protein extraction and peptides separation

## **3 Optimisation of protein extraction and peptides separation**

### **3.1 Introduction**

#### **3.1.1 Proteins extraction approaches**

Proteomics aims to study the whole protein content of a specific biological sample regardless of the tremendous variation in their abundance and biophysical properties. However, proteomics data depends qualitatively and quantitatively on the proteome coverage which can be extended mainly by increasing the quality and quantity of extracted proteins as well as improving the resolving power of protein separation techniques.

Protein extraction methods are a preliminary step in the proteomics workflow, which can be conducted for protein analysis such as protein quantitation and/or identification, as well as for studying protein functions. Indeed, the protein extraction process involves two key steps: cell or tissue disrupt and protein solubilisation. However, extracting the comprehensive protein populations from biological tissues, such as liver, can be particularly challenging due to their high degree of complexity and heterogeneity.

In that respect, a number of extraction methods for proteins from soft tissues, such as the brain, liver, and kidney, however, have been developed and require relatively gentle techniques compared to hard tissues (e.g., skeletal and cardiac) which are more susceptible to harsh shear forces (Skehel, 2004). These methods range from the simplest (e.g., enzymatic digestion and osmotic shock) to the moderate (e.g., ultrasonication) and to the harsh (e.g.,

mechanical blender and homogenizer) (Grabski, 2009). Generally, different extraction methods can result in extracting different classes of proteins when applied on the same sample. For example, methods in which detergents are omitted will primarily result in extracting the cytosolic proteins. (Burden, 2012a).

Choosing the appropriate extraction method depends mainly on the type of sample, cellular location of targeted proteins and the structure of desired proteins (intact vs denatured). For example, heat generating or protein denaturing methods must be avoided in studies concerning enzyme activity (Burden, 2012a).

Chemical based extraction method tends to involve different kinds of detergents, organic buffer or organic acid for cellular membrane disruption, breaking lipid-protein interactions and solubilising the proteins, as shown in Table 1.3. (Shevchenko et al., 2012). Sodium dodecyl sulphate (SDS) is a common example of anionic detergents which nowadays are used extensively in proteins extraction and able to solubilise a wide range of proteins (Scheerlinck et al., 2015). However, although a detergent like SDS is relatively cheap, easy to use and has available proved and effective protocols, it can negatively affect the proteomics analysis at several levels such as; (i) digestion level by denaturing trypsin enzyme, (ii) the resolving power of RP-HPLC in peptides separations and (iii) the MS analysis level by suppressing peptides ionisation and masking peptide-derived signals by generating intense background signals (Lu and Zhu, 2005, Ernoult et al., 2008, Hustoft et al., 2011, von Hagen, 2011). In addition, at a protein functional level, detergents can result in denaturing the three-dimensional structure of proteins causing

them to lose their activity (Shevchenko et al., 2012). Although Shevchenko and co-workers have found that detergents such as SDS can be cleaned up by filter-aided sample preparation (FASP) method, it has been suggested that FASP was not efficient enough to remove all SDS traces, in addition to being time-consuming (Shevchenko et al., 2012).

On the other hand, mechanical-based extraction methods (Table 1.4) have proven their ability to extract a remarkable amount of proteins by applying shear forces to breakdown the cellular membrane and emancipate the inner cellular proteins to be dissolved subsequently with a suitable hypotonic buffer (Burden, 2012a). However, using mechanical-based methods have some challenges to be considered. For example, some of the mechanical systems are expensive, ponderous and variable in performance making protocols difficult to replicate (e.g. different fitting size of homogenization pestles). Another difficulty is the heat generation, which may cause proteins denaturation and aggregation though this can be overcome by pre-chilling the equipment as well as keeping the sample in ice throughout preparation (Burden, 2012a).

### **3.1.2 Proteins separation approaches**

Shotgun proteomics approach is a powerful technique for biomarker candidates' discovery that is based on combining two techniques for tryptic peptides separation in two dimensions' format. Throughout proteomics study, protein separation is the most laborious task since it must cope with a myriad of various properties and characteristic to each protein, such as molecular weight and isoelectric point (pI).

One of the major limitations for proteomics is the low resolving power in the recovery of hydrophobic proteins. For example, membranous proteins cannot be negligible since they represent about 30% of the typical proteome, and since approximately 70% of the available drugs targeting proteins are active against this class of proteins (Perez-Reyes et al., 1990, Tan et al., 2008). Another limitation is the wide dynamic range of proteins concentration in tissues, extending from 7 and up to 10 orders of magnitude, which prevents identification of low abundant proteins (Dwane and Kiely, 2011). Therefore, it has been a necessity to develop separation techniques that allow a significantly higher proteome coverage in the sample, which will ensure the identification of the low abundant proteins and thus a comprehensive analysis of complex samples (Ernault et al., 2008).

Isoelectric-focusing was implemented as a first dimension separation technique for the first time in 2004 in shotgun proteomics using an OFF-GEL (OG) system (Cargile et al., 2004b). OG separates proteins/peptides depending on their isoelectric points (pIs). As a consequence of moving proteins and peptides over a pH gradient gel, their net charges will change till becoming null at a specific point and are no longer attracted by the electrical current. At this point, proteins/peptides become focused according to their pI within the pH gradient (Cargile et al., 2004b). However, IEF for protein separation is considered less suitable, compared to peptides, as proteins tend to precipitate at their pI, reducing protein recovery from the gel media (Wall et al., 2000).

Currently, ion exchange chromatography and thus SCX is the traditional and the most commonly used approach for the first-dimensional separation

followed by RP-HPLC as the second dimension of separation (Puangpila et al., 2015). SCX is a well-established method that has proved its ability to work over a wide range of pH as well as to be coupled directly to RP-HPLC (on-line) in a shotgun approach due to the limited resolution (Dai et al., 2009, Di Palma et al., 2012a). However, It has been reported that using SCX as purification step has led to reducing ion suppression caused by detergents or chemicals from iTRAQ reagents (Kong et al., 2011). On the other hand, OG has been considered as the best alternative for SCX fractionation for first dimension separation in a shotgun approach due to its high reproducibility and resolving power, making it essential for membranous proteins analysis (Chick et al., 2008). In addition, OG has been considered superior to other separation methods due to its great loading capacity (up to 5mg of proteins/peptides), and its ability to provide additional information that can be used as a filtration criterion to reduce false positive and false negative identifications. Therefore, this will improve confidence in protein and PTMs identifications (Millioni et al., 2013, Krijgsveld et al., 2006). In contrast to SCX, OG has been considered as an efficient method that is capable to separate both basic and acidic peptides by using the advancement of focused immobilized pH gradient (IPGs) strips, which enables flexibility in selecting the preferred pH gradient, such as those peptides clustered at pH 3 (Krijgsveld et al., 2006, Eriksson et al., 2008).

For quantitative proteomics experiments, IEF has proved its compatibility with peptide tagging reagents such as iTRAQ since the functional groups of tags are not charged and do not significantly influence focusing of tagged peptides (Lengqvist et al., 2007).

### **3.1.3 Aims of the chapter**

This chapter was conducted to optimise two elementary steps of shotgun proteomics workflow in two parts. The optimised methods from this chapter will be applied for subsequent proteomics experiments.

- a. Using total liver extracts, rather than the microsomal fraction, the first part of this chapter aims to determine:
  - The effect of freezing during storage, on the quantity of the extracted protein.
  - The most efficient mechanical method in protein extraction (cryo-pulverization CP, cryo-pulverization and sonication CP+S, disperse homogenizing H and liquid Dounce homogenizing D) as determined by protein yield using the Bradford assay.
  - The effect of each mechanical method on mouse liver proteome, particularly on drug metabolizing enzymes (Phase I and II) by a shotgun proteomics approach.
- b. The second part of this chapter aims to determine:
  - The optimal peptides separation method using two well-established methods (OG and SCX)
  - The effect of each separation method on mouse liver proteome qualitatively and quantitatively, particularly on drug metabolizing enzymes (Phase I and II).



## **3.2 Animals and Method**

### **3.2.1 Animal Maintenance**

BALB\cOLaHsd –Foxn1nu immunodeficient nude mice (Harlan Laboratories, UK) aged 10 weeks were used. Mice received CRM diet (S.D.S., Witham, UK) and water ad libitum. Mice were kept in cages in an air-conditioned room with regular alternating light and dark cycles. All animal procedures were carried out under a project licence issued by the UK Home Office and following the UKCCCR guidelines (Workman et al., 1998).

### **3.2.2 Sample Preparation**

#### **3.2.2.1 Mouse Liver Protein Extraction**

Normal liver tissues were initially weighed and extracted by one of four mechanical methods, (Figure 3.1) using a freshly prepared phosphate buffered saline (PBS) containing complete mini EDTA-free protease inhibitor cocktail tablet (one tablet for 7 ml PBS) as a buffer. Tissues were kept in ice during protein extraction processes.

##### **3.2.2.1.1 Cryo-pulverization method (CP)**

The stainless steel Cell crushes Tissue Pulveriser (Cellcrusher, Cork, Ireland) consists of a two-component mortar with handles and a pestle, specifically designed for pulverizing 10 - 1000mg of tissue. Liver tissue homogenization was carried out as previously described (Toivonen et al., 2014, Lesseur et al., 2014, Alhamdani et al., 2010). Briefly, after chilling the pulveriser and the liver sample in liquid nitrogen, the lead hammer was used on the mortar to

completely fracture the chilled sample. After that, the crushed sample was transferred to a 50 ml Falcon tube containing freshly prepared phosphate buffered saline (PBS) with a complete mini EDTA-free protease inhibitor cocktail tablet (one tablet for 10 ml PBS). The homogenate was then centrifuged for 30 minutes at 9,000 *g* at 4°C; the supernatant was collected and aliquoted into new Eppendorf tubes and kept at -20°C for further analysis.

#### **3.2.2.1.2 Combination of cryo-pulverization and Sonication method (CP+S)**

Optionally, after pulverizing the liver sample, sonication was carried out using Philip Harris Scientific Sonicating probe, (Scientific Laboratory Supplies Ltd, UK). The suspended sample was sonicated for 6 cycles, 5 seconds for each (tissue was cooled on ice for 20 seconds in between). Homogenates were centrifuged for 30 minutes at 9,000 *g* at 4°C. The supernatant was collected and stored at -20°C for further use as described above.

#### **3.2.2.1.3 Disperse homogenizer method (H)**

The liver sample was immersed in freshly prepared phosphate buffered saline (PBS) containing complete mini EDTA-free protease inhibitor cocktail tablet (one tablet for 10 ml PBS). The sample was homogenized whilst kept in ice for 5 times, 30 seconds for each using a disperse Ultra-Turrax T25 homogeniser (Janke and Kunkel, IKA Labortechnik, Staufen, Germany) as described previously (Alhamdani et al., 2010). Homogenates were centrifuged for 30 minutes at 9,000 *g* at 4°C. The supernatant was collected and kept at -20°C as described previously.

#### 3.2.2.1.4 Liquid Dounce homogenizer method (D)

According to a previous report (Burden, 2008), liver tissue was chopped into small pieces. Approximately 1 ml of freshly prepared phosphate buffered saline (PBS) containing complete mini EDTA-free protease inhibitor cocktail tablet (one tablet for 10 ml PBS) was mixed with small pieces of chopped tissue for homogenizing by Tenbroeck-Dounce Tissue Grinder (Wheaton, USA). Chopped tissue was broken up with 10 to 12 plunges until no large pieces remain. Homogenates were collected in 50 ml Falcon tube and centrifuged for 60 minutes at 9,000 *g* at 4°C. After that, the supernatant was collected and kept at -20°C as described previously.

#### 3.2.2.2 Protein digestion and iTRAQ Labelling

Protein extracts from each method were quantified using Bradford method, then protein, equivalent to 50 µg, precipitated by chilled acetone (sections 2.2.1.3-4). Samples were then digested by trypsin (section 2.2.1.5); the resultant peptides were purified and desalted prior iTRAQ labelling (section 2.2.1.6-7). The iTRAQ was processed as shown in Table 3.1, with the homogenizing method (H) as the control.

**Table 3-1. 4-plex iTRAQ reagents used to label protease digests of mouse livers extracted with different mechanical extraction method.**

iTRAQ reagent	114	115	116	117
Extraction method	CP	CP+S	H	D

### 3.2.2.1 Peptides separation and protein identification

The combined iTRAQ sample was divided into two halves. Each half was separated in the first dimension using either SCX (12 flow-through and elution fractions) or OG (12 cm IPG strip, 12 Well Frame Set and 3-10 pH) method as described in Sections 2.2.1.8.1.1-2. Then, the resultant fractions (12 fractions for each method) were desalted and separated for their second dimension using RP-HPLC method (section 2.2.1.8.2). The MS analysis and proteins identifications were conducted as described in Sections 2.2.1.9.

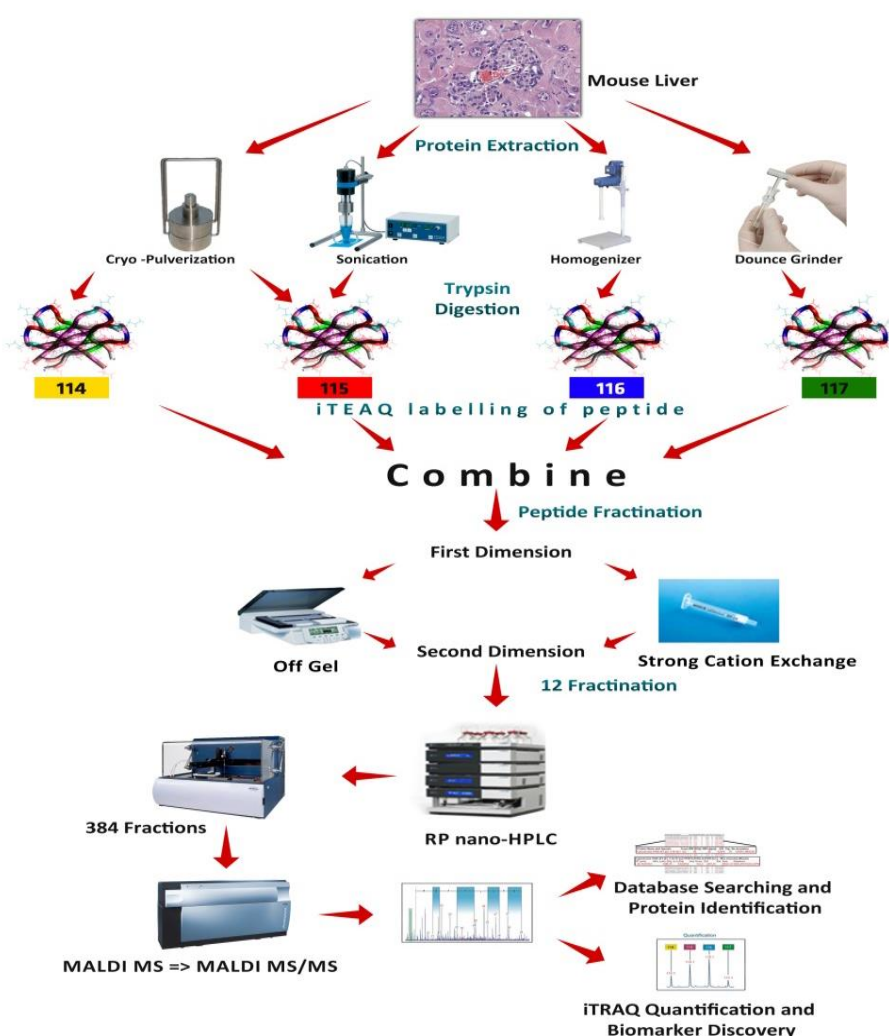
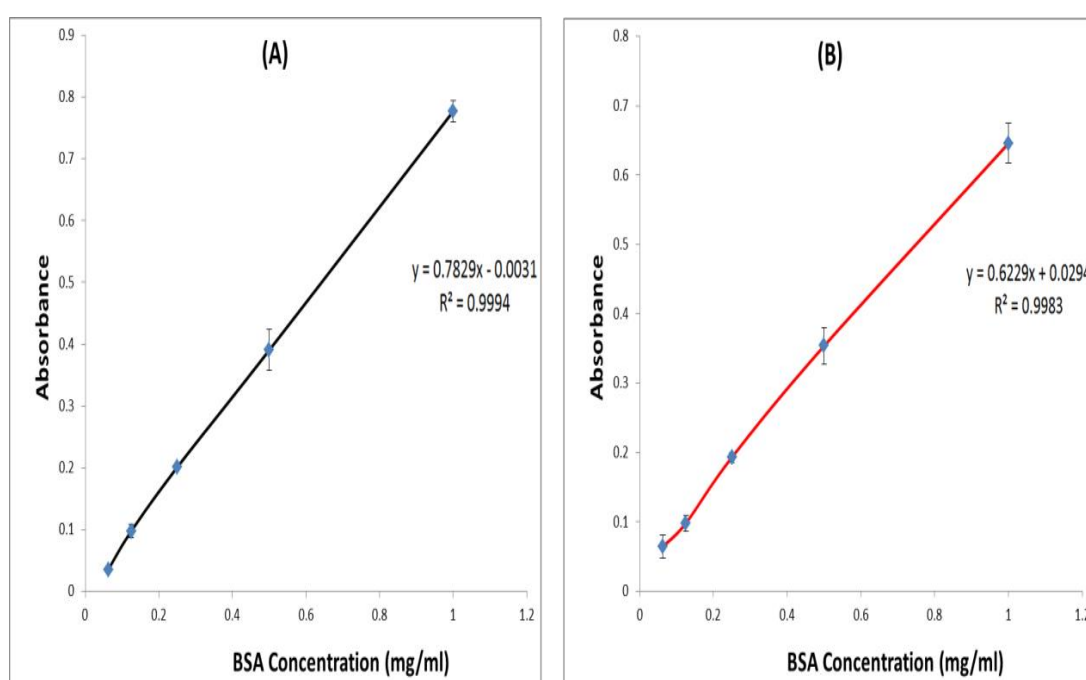


Figure 3-1. Quantitative proteomics workflow shows the four mechanical extraction methods, iTRAQ labelling, the two-dimensional separation techniques, protein identifications by MALDI-MS and database searching.

## 3.3 Results

### 3.3.1 Protein extraction optimisation by Bradford assay

In order to optimise protein extraction by mechanical methods and determine the best one, frozen and fresh normal mouse liver tissues were subjected to protein extraction by four mechanical methods (cryo-pulverization - CP, cryo-pulverization, and sonication - CP+S, disperse Homogenizing - H, and liquid Dounce homogenizing - D) in two duplicate experiments. The extracted protein concentration was determined using Bradford assay (section 2.2.1.3). Standard curves were created for frozen and fresh samples (Figure 3.2).



**Figure 3-2. Standard curves of BSA for proteins quantitation for (A) Frozen and (B) Fresh livers.**

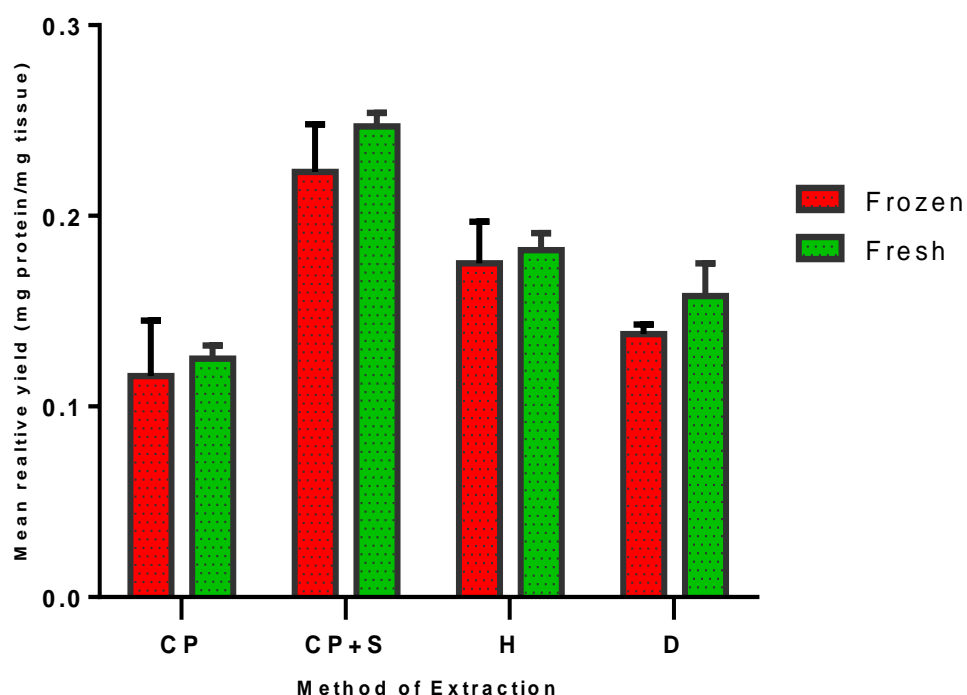
The four extraction absorbance values were extrapolated to standard curves to determine the amount of total extracted proteins and then the protein-to-tissue ratio, for frozen and fresh mouse livers (Table 3.2).

**Table 3-2. Comparison of four mechanical methods for the extraction of the total proteins from frozen and fresh mouse liver**

Sample condition	Method of extraction	Mean relative yield (mg protein/mg tissue)	Standard deviation	Coefficient of variation (CV %)
<b>Frozen</b>	CP	0.116	0.029	24.7
	CP+S	0.223	0.025	11.0
	H	0.173	0.022	12.4
	D	0.138	0.005	3.7
<b>Fresh</b>	CP	0.125	0.007	5.6
	CP+S	0.247	0.007	2.8
	H	0.182	0.009	4.8
	D	0.158	0.017	10.5

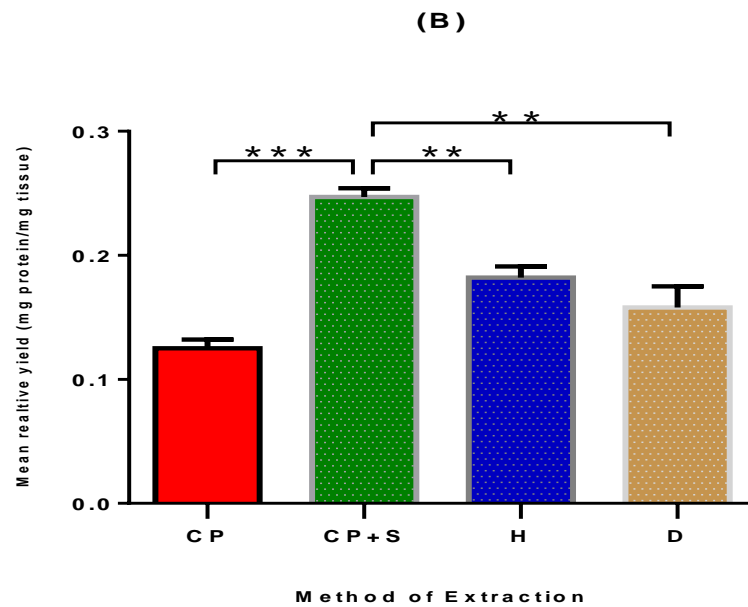
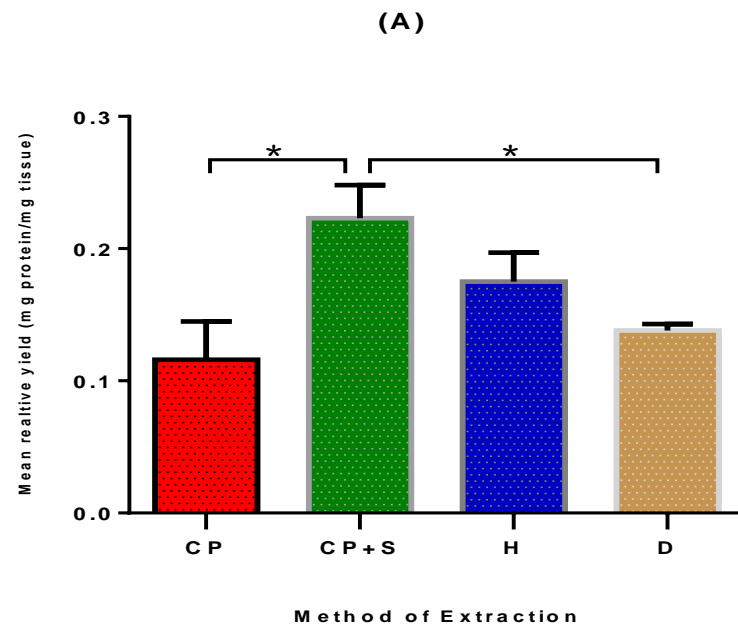
Furthermore, to explore whether there are any differences between frozen and fresh liver samples, the amount of protein in mg per mg of tissue for each extraction method was presented for frozen and fresh mouse livers as shown in Figure 3.3.

The result demonstrated that there are no significant differences between frozen and fresh samples in the amount of extracted protein-to-tissue ratio for each extraction method. However, the results indicate that the CP+S method produced the highest yield of proteins.



**Figure 3-3. Comparison of the amount of extracted proteins (mg) to tissue (mg) between frozen and fresh mouse livers for each extraction method**

To confirm that CP+S was a significant improvement compared to the others a one-way ANOVA test was performed. The combination of cryo-pulverization and sonication method (CP+S) has generated the maximum yield of proteins with 0.223 and 0.247 (mg/mg protein-to-tissue ratio) for both frozen and fresh samples, respectively (Figure 3.4). Moreover, the results confirmed statistically that CP+S method is superior compared to CP, H and D methods for frozen and fresh samples apart from the comparison of CP+S and H extraction methods of the frozen liver.



**Figure 3-4. Comparison the four extraction methods for their amount of extracted protein-to-tissue ratio within each sample condition. (a) Frozen sample and (b) Fresh sample. The significant difference between CP+S method and other methods is indicated with asterisks (p-value < 0.05).**

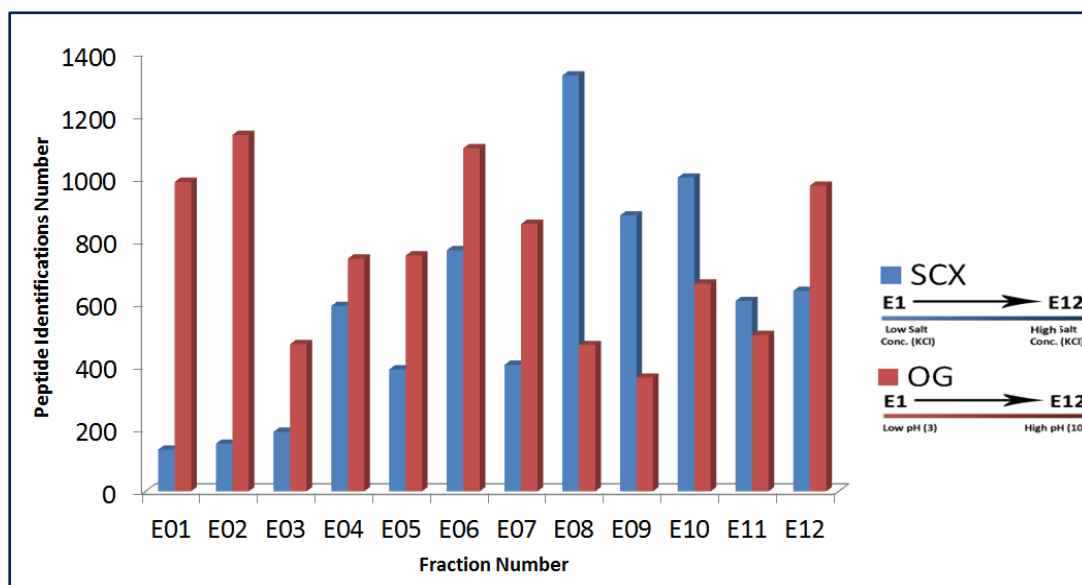


### **3.3.2 Protein separation optimisation by quantitative proteomics**

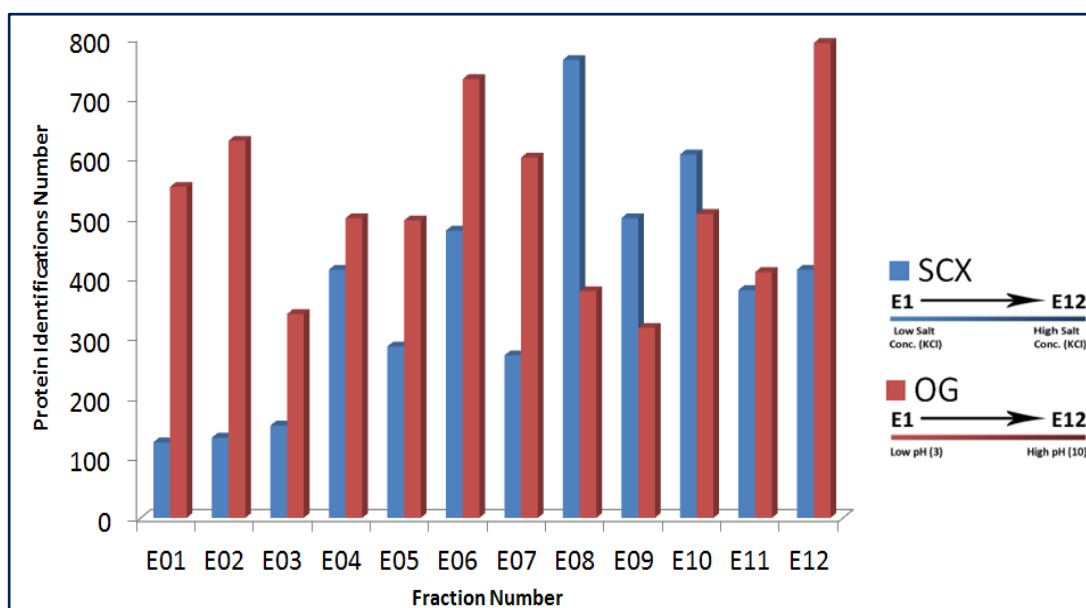
Two first dimension separation techniques were evaluated: OFF-GEL (OG) and strong cation exchange (SCX), as part of a comparative proteomics study. Identified proteins and peptides were compared in order to determine which technique provides the highest performance for analysis of a complex mixture of tryptic peptides extracted from fresh mouse liver, by one of four mechanical methods (CP, CP+S, H, and D). An equal number of fractions (12 in each case) were collected by SCX chromatography using a serial concentration of elution buffer (30-1000 mM KCl) or by isoelectric-focusing (IEF) on OG (pH 3-10, 12cm IPG strip). The second dimension was kept constant; i.e. reverse phase HPLC separation. The amount of sample used in each separation method was also kept constant. In addition, database searching was performed using the same parameters.

#### **3.3.2.1 Identified proteins analysis**

Data analysis indicated that a total of 6254 proteins and 9010 peptides were identified by OG method (Fractions 1 -12), with an average of 521 proteins per fraction (SD=149) and 751 peptides per fraction (SD=264). Whereas 4528 total proteins and 7089 total peptides, average of 377 proteins per fraction (SD= 196) and 591 peptides per fraction (SD=366) were extracted from 12 fractions by SCX. Peptides and proteins distribution is shown in Figures 3.5 and 3.6, respectively.



**Figure 3-5. The number of identified peptides for OG and SCX methods per each fraction.**

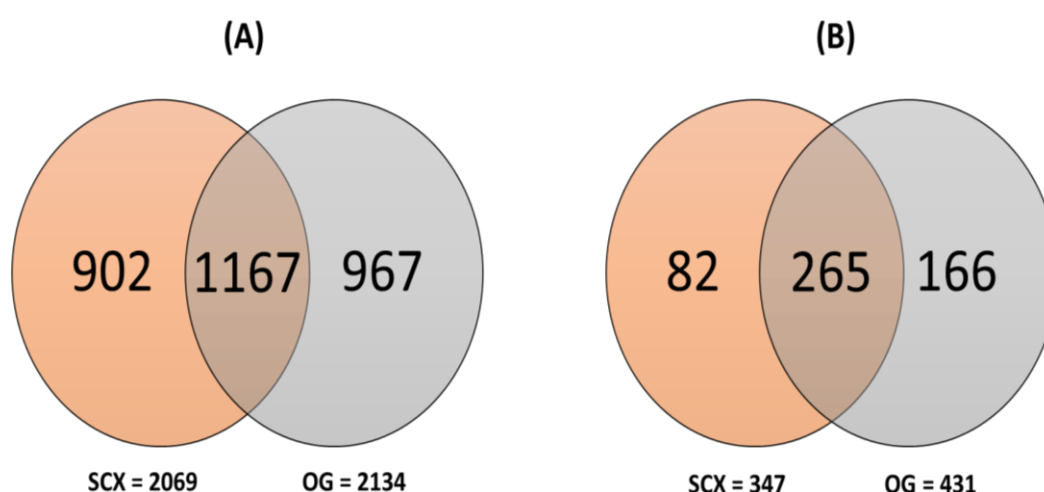


**Figure 3-6. The number of identified proteins for OG and SCX methods per each fraction.**

For peptide identification, SCX showed greater variability across the 12 fractions, with most of the peptides eluted in fractions number 6, 8, 9 and 10, demonstrating SCX was highly sensitive to pH differences. However, in OG

method, the identified peptides were distributed more evenly, since a wide pH strip was used. At the protein level, the same results were found regarding the distribution of the identified proteins by SCX and OG methods.

Unambiguously identified proteins in each fraction were combined into a non-redundant list as described in Section 2.2.1.10 for OG and SCX (Appendix AI and AII, respectively). There are 2265 peptides (2134 unique peptides) and 2222 peptides (2069 unique peptides), resulting in 431 and 347 proteins identified by OG and SCX method, respectively. Of these, 1167 peptides and 265 proteins were common between OG and SCX (identified by both methods) as summarized by Venn diagrams in Figure 3.7.

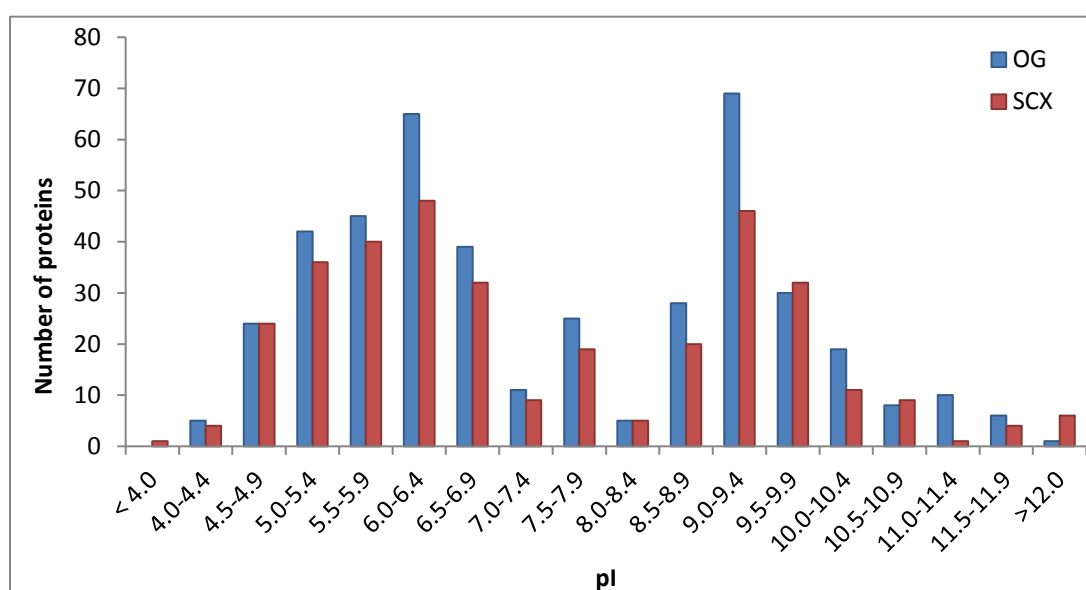


**Figure 3-7. Venn diagrams comparing the total and unique numbers of unambiguously identified (A) peptides and (B) proteins.**

For unique peptide identifications, the results demonstrate that 1167 peptides were confidently identified by both methods. However, OG resulted in the identification of more peptides (2134) in comparison to SCX (2069). At the protein level, 431 and 347 proteins were identified by OG and SCX

respectively, among which 265 proteins were detected in both methods representing 68.2% of all proteins detected by both methods when combined.

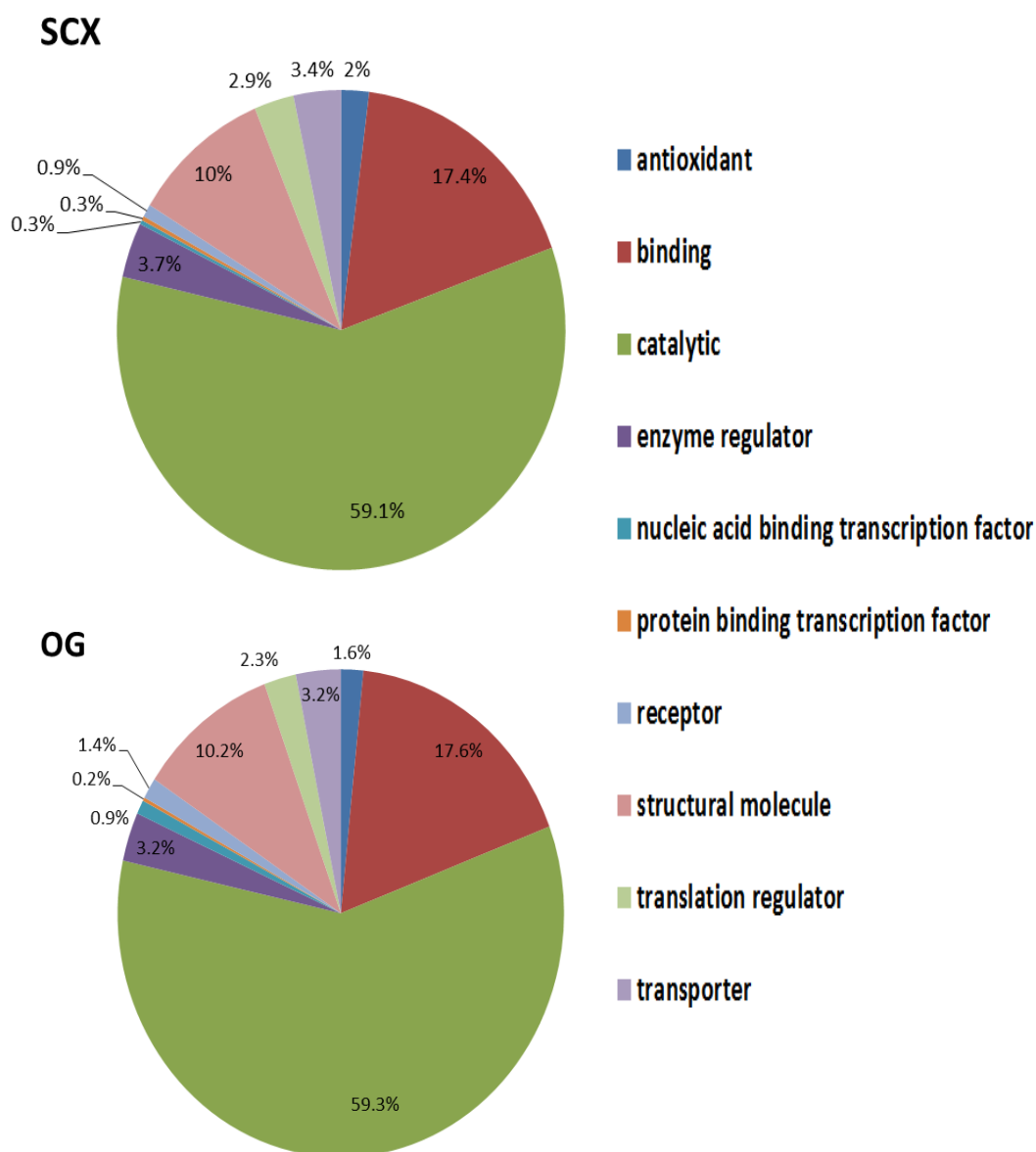
Comparison between the theoretical isoelectric points (pI) for identified proteins from OG and SCX methods (Figure 3.8) revealed that both methods are able to identify proteins over a wide range of pH. However, in both methods, approximately 48% of identified proteins have pI equal to 7 or higher, indicating that these two methods can be used for detecting alkaline proteins, similar to the other traditional method 2D gel electrophoresis. But, proteins with pI higher than 10, which are not readily detected by 2D-PAGE, represented 10.4% and 8.9% of total proteins identified by OG and SCX, respectively.



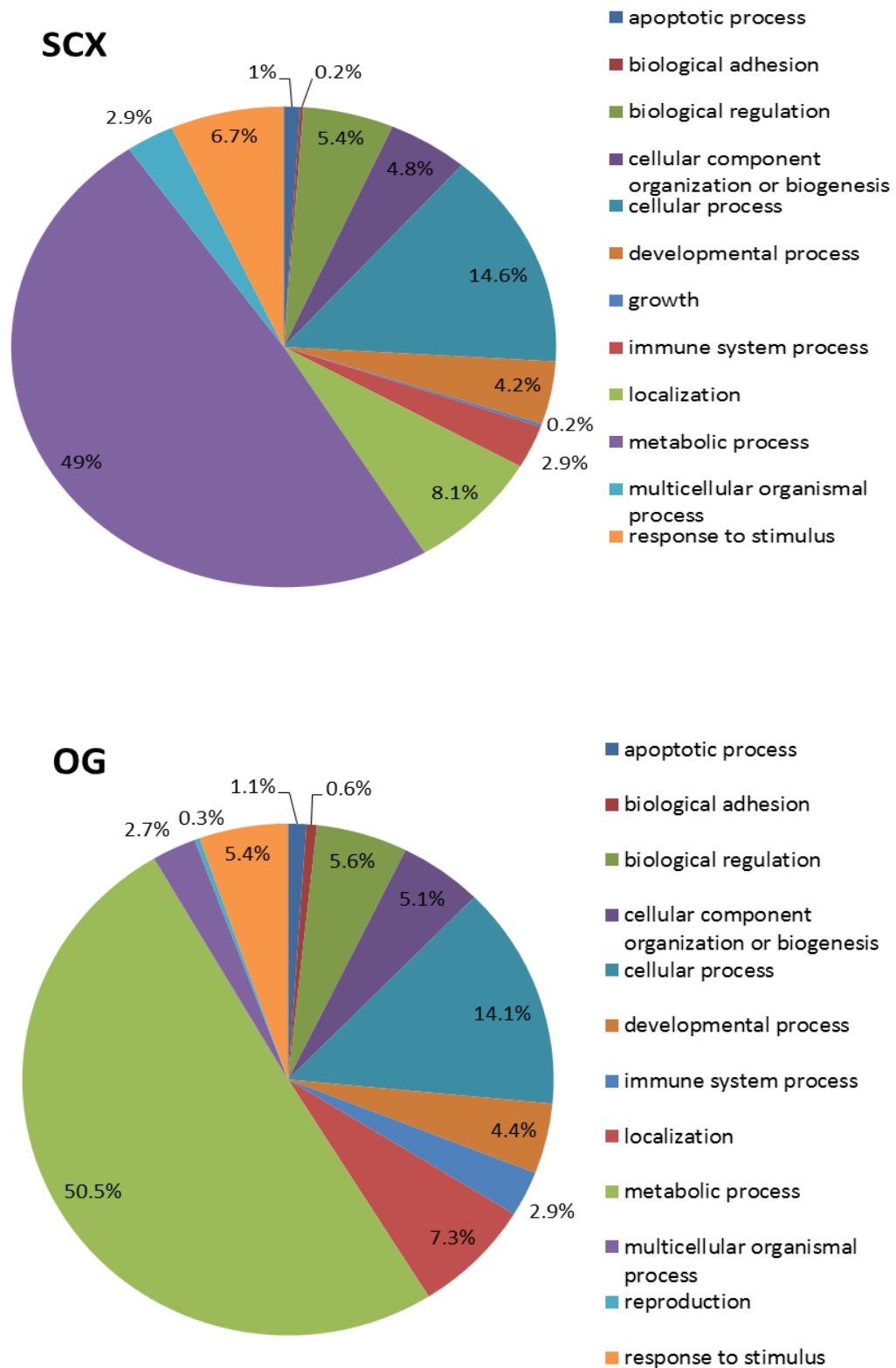
**Figure 3-8. Distribution of identified proteins by OG and SCX over the isoelectric point (pI) values.**

Further classification of confidently identified proteins by gene ontology (GO) into different categories based on their molecular functions and biological

processes was also performed using PANTHER tool (section 2.2.1.13) (Figure 3.9 and Figure 3.10, respectively).



**Figure 3-9. Gene ontology for identified proteins by SCX, n=347 (above) and OG, n=431 (below) based on their molecular function. Proteins were classified into 10 groups, where the percent of each group represent the relative abundance.**



**Figure 3-10. Gene ontology for identified proteins by SCX, n=347 (above) and OG, n=431 (below) based on their biological processes. Proteins were classified into 12 groups, where the percent of each group represent the relative abundance.**

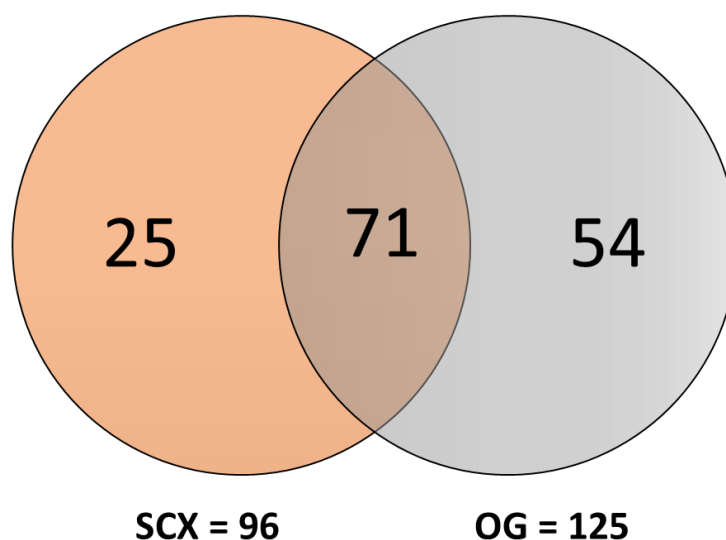
GO analysis classified the identified proteins from SCX and OG into 10 groups based on their molecular functions (Figure 3.9), with the highest proportion being involved in a catalytic activity for both SCX (59.1%) and OG (59.3%) proteins, followed by binding and structural molecule activity. The results demonstrated that there was no significant variation in the relative abundance of each group of proteins for the molecular function between SCX and OG proteins.

When classified by biological processes (Figure 3.10), 12 groups were identified with a major contribution representing the metabolic process for SCX (49%) and OG (50.5%). This provided clear evidence that liver is highly enriched in metabolic enzymes. However, it was seen that growth proteins (1%), which have a role in cell or organism proliferation (e.g. Epidermal growth factor receptor), and reproduction class (0.3%), which have a role in sexual reproduction such as spermatogenesis (e.g. Estradiol 17 beta-dehydrogenase 5), were unique for SCX and OG, respectively.

Moreover, it was seen that a slightly higher proportion of the SCX proteins were involved in cellular localization and response to stimulation processes, while more proteins were associated with metabolic processes from OG proteins. Many of these metabolic process proteins play an essential role in detoxification of xenobiotics, such as; glutathione S-transferase enzymes (GSTs), cytochrome P450 enzymes (CYP450s), major urinary proteins (MUPs), aldehyde dehydrogenase enzymes (ALDHs), and others.

Next, in order to evaluate the power of OG and SCX methods in resolving the hydrophobic proteins (membranous proteins), a preliminary gene ontology

analysis (section 2.2.1.13) was conducted according to the reported annotation in the UniProt™ database and only protein annotated to a single membranous class was included (Figure 3.11).



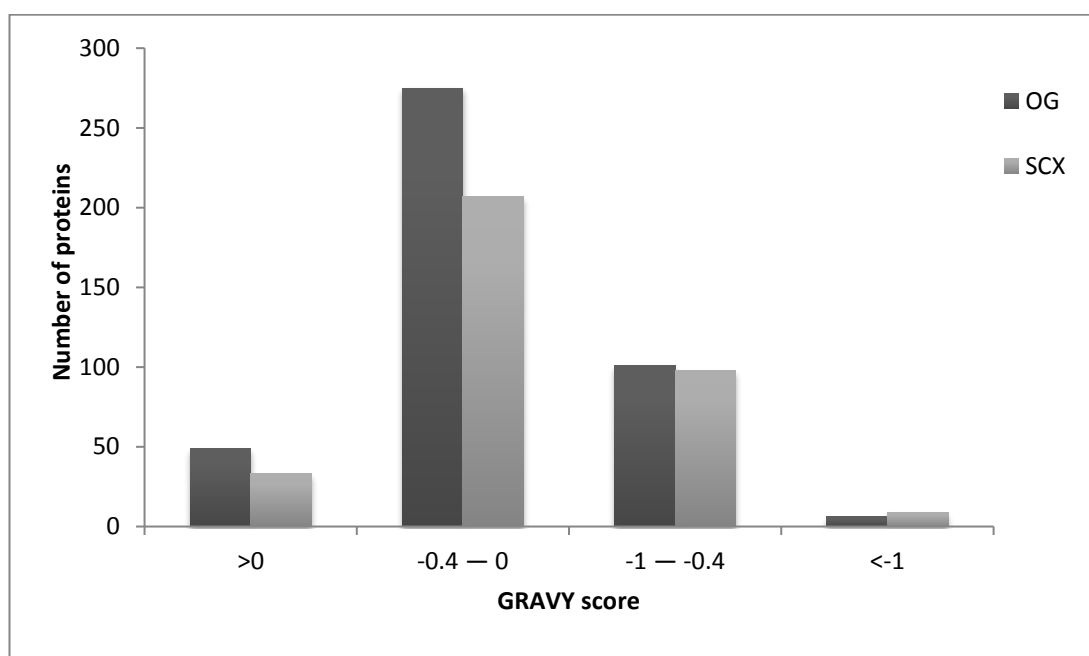
**Figure 3-11. Venn diagram for proteins which are annotated as membrane proteins for OG and SCX method.**

A total of 125 and 96 proteins were annotated to the membranous fraction by OG (29.1%) and SCX (27.6%), respectively. Of the membranous proteins, 71 proteins were identified by both methods, whereas 54 and 25 were uniquely detected using the OG and SCX methods, respectively. Findings indicated that OG method was superior in resolving membranous proteins.

Moreover, the grand average of hydropathy (GRAVY) score was calculated for identified proteins in order to evaluate the hydrophobic status of proteins (section 2.2.1.13).



In the current comparison study, a total of 324 (75.2%) proteins and 240 (69.2%) proteins identified from OG and SCX had GRAVY score of higher than (-0.4), suggesting membrane proteins (Figure 3.12).



**Figure 3-12. Distribution of identified proteins from OG and SCX over the GRAVY scores. The value (-0.4) is the threshold between cytosolic proteins and membrane proteins, therefore protein with a score higher than (-0.4) indicates a higher probability for membrane association (a more positive GRAVY score the more hydrophobic a protein).**

The results demonstrated that both methods were able to recover membrane proteins. However, GO analysis and GRAVY score indicated that OG is superior for hydrophobic proteins recovery from mouse liver extracts. Thought that GO analysis included only proteins with a single annotation to membrane class, which explains the differences in the percentage of membranous proteins between two methods.

### **3.3.2.2 Qualitative analysis of Cytochrome P450 enzymes extracted by OG and SCX**

Since CYP450 family is a key example of the membrane proteins in the liver and important in the metabolism of drugs, CYP450 subfamily enzymes were investigated qualitatively and quantitatively among the four mechanical methods (CP, CP+S, H, and D).

Due to the high degree of sequence homology within the sub-families' isoforms, CYP isoform was considered significantly identified according to criteria described previously in Section 2.2.1.12. Unique peptides identification and homology of closely related CYP isoforms determination were performed using (Skyline 3.5-64 bit) software and CLUSTAL O (1.2.1) tools (section 2.2.1.13).

An example of determining whether the peptide is unique or common with other close isoforms and subsequently deciding if that CYP isoform is significantly identified is shown in Figure 3.13. Based on these criteria, seven CYP450 enzymes (CYP1a2, 2c70, 2d9, 2d10, 2d26, 2e1, and 2f2) were identified by OG and four (CYP2c70, 2d9, 2d10 and 2e1) were identified by SCX (Table 3.3). The results demonstrated that OG was superior for Cytochrome P450 enzymes identification. Although SCX showed a slight improvement in unique peptides identified, as well as sequence coverage for CYP isoforms, it was unable to separate and detect CYP1a2 which is a highly abundant isoform.

In conclusion, observing a higher number of CYP identifications than previously reported was not expected, since S9 fractions (whole liver extracts)

were used for this optimisation rather than microsomal fractions, which enables enrichment of metabolic enzymes.

<u>CYP isoform</u>	<u>Sequence</u>
CP2DQ_MOUSE	MGLLVGDDLWAVVIFTAIFLLLVDLVHRRQRWTACYPPGPVFPFGLGNLLQVDFENIPYS
CP2D9_MOUSE	MELLTGTDLWPVAIFTVIFILLVDLTHQRQRWTSRYPPGPVFPVPLGNLLQVDLGNMPYS
CP2DA_MOUSE	MELLTGAGLWSVAIFTVIFILLVDMHRHQRWTSRYPPGPVFPVPLGNLLQVDLDNMPYS
CP2DB_MOUSE	MELLTGAGLWSVAIFTVIFILLVDMHRHQHWTSCPPGPVFPVPLGNLLQVDLGNMPYS
CP2DQ_MOUSE	FYKLQNRYGNVFSLQMAWKPVVVVNLKAVRELLVTYGEDTSRPLMPIYNHIGYGHKSK
CP2D9_MOUSE	LYKLQNRYGDVFSLQMAWKPMVVINGLKAMKEMLLTCGEDTADRPPVPIFEYLGVKPGSQ
CP2DA_MOUSE	LYKLQNRYGDVFSLQMGWKPMVVINGLKAMKEVLLTCGEDTADRQVPIFEYLGVKPGSQ
CP2DB_MOUSE	LYKLQNRYGDVFSLQMGWKPMVVINGLKAMKEVLLTCGEDTADRQVPIFEYLGVKPGSQ
CP2DQ_MOUSE	GVVLAPYGPEWREQRRFSVSTLRD FGLGKKSLEQWVTEEAGHLCD AFTKEAEHPFNPSPL
CP2D9_MOUSE	GVVLAPYGPEWREQRRFSVSTLRN FGLGKKSLEDWVTKEANHLCD AFTAQAGQPINPNPM
CP2DA_MOUSE	GVVLAPYGPEWREQRRFSVSTLRN FGLGKKSLEDWVTKEARHLCD AFTAQAGQPINPNTM
CP2DB_MOUSE	GVVLAPYGPEWREQRRFSVSTLRN FGLGKKSLEDWVTKEARHLCD AFTAQAGQPINPNTM
CP2DQ_MOUSE	LSKAVSNVIASLIYARRFEYEDPFFNRMLKTLKESLGEDTG FVGEVLNAIPMLLHIPGLP
CP2D9_MOUSE	LNKSTCNVIASLIFARRFEYEDPFLIRMLKVLEQSLTEVSGLIPEVLNAFPILLRIPRLA
CP2DA_MOUSE	LNNAVCNVIASLIFARFEEYEDPYLIRMQKVLEDSLTEISGLIPEVLNMFPIILLRIPGLP
CP2DB_MOUSE	LNNAVCNVIASLIFARFEEYEDPYLIRMLKMLKECFTEISGFIPGVLNEFPFIPLRIPGLA
CP2DQ_MOUSE	DKAFFPKLSNFIALVNMKMLIEHDLTWDPAQPPRDLTDAFLAEVEKAKGNPESSFNNDKNLRI
CP2D9_MOUSE	DKALQGQKSFIAILDNLLTENRTTWDPVQAPRNLDAFLAEIEKAKGNPESSFNNDENLLM
CP2DA_MOUSE	GKVFQGGKSLLAIVENLLTENRNTWDPDQPPRNLDAFLAEIEKVKGNAESSFNNDENLRM
CP2DB_MOUSE	DMVFQGGKSFMAILDNLLTENRTTWDPDQPPRNLDAFLAEIEKAKGNPESSFNNDENLRM
CP2DQ_MOUSE	VVIDLFMAGMVTSTTSLWALLMILHPDVQRRVHQEIDEVIGHVRHPEMADQARMPYTN
CP2D9_MOUSE	VVRDLFGAGMLTTSTTSLWALMLMILHPDVQRRVQQEIDEVIGQVRHPEMADQAHMPYTN
CP2DA_MOUSE	VVLDLFTAGMVTSTTSLWALLMILHPDVQRRVQQEIDAVIGQVRHPEMADQARMPYTN
CP2DB_MOUSE	VVGDLFTAGMVTSTTSLWALLMILHPDVQRRVQQEIDAVIGQVQHPEMADQARMPYTN
CP2DQ_MOUSE	AVIHEVQRFADIVPTNLPHMTSRDIKFQDFFIPKGTTLIPNLSSVLKDETVWEKPLRFYP
CP2D9_MOUSE	AVIHEVQRFQDIPVNLPRITSHDIEVQDFLIPKGTILLPNMSSMLKDES VWEKPLRFHP
CP2DA_MOUSE	AVIHEVQRFQDIAPLNLPRITSRDIEVQDFLIPKGSILIPNMSSVLKDETVWEKPLRFHP
CP2DB_MOUSE	AVIHEVQRFQDIAPLPLPRITSRDIEVQDFLVTKGSTLIPNMSSVLKDETVWEKPLRFHP
CP2DQ_MOUSE	EHFLDAQGHFVKHEAFMPFSAGRRSCLGEPLARMELFLFFTCLLQRF SFSVPDGGQPRPSD
CP2D9_MOUSE	EHFLDAQGHFVKPEAFMPFSAGRRSCLGEALARMELFLFFTCLLQRF SFSVPDGGQPQPSN
CP2DA_MOUSE	EHFLDAQGHFVKPEAFMPFSAGRRSCLGEPLARMELFLFFTCLLQHFSFSVPNGQPRPRN
CP2DB_MOUSE	EHFLDAQGHFVKPEAFMPFSAGRRSCLGEALARMELFLFFTCLLQRF SISVPDGGQPQPSN
CP2DQ_MOUSE	YGIYTMPVTPEPYQLCAVAR----
CP2D9_MOUSE	SGVYGILVAPSPYQLCAVVRDQGH
CP2DA_MOUSE	LGVFPPFPVAPYPYQLCAVMREQGH
CP2DB_MOUSE	YRVHAIPVAPFPYQLCAVMREQGH

**Figure 3-13. An example of sequence alignment for a closely related subfamily CYP2D (CP2DQ = CYP2D26, CP2D9 = CYP2D9, CP2DA = CYP2D10, CP2DB = CYP2D11) identifying common and unique peptides determination. Red sequence indicates a unique peptide for a specific CYP, where blue sequence indicates a common peptide among the different isoforms. Sequence alignment was created by Clustal Omega tool.**

**Table 3-3. Cytochrome P450 enzymes unambiguously identified by OG and SCX, their average MASCOT score and the number of common and unique peptides. CYP isoforms were defined by at least one unique peptide with significant MASCOT score. ND indicated not detected for SCX method.**

				OG					SCX					
CYP isoform	Uniprot/ accession number	M.Wt (KDa)	pI	Total number of peptides	Number of unique peptides	Number of spectra for unique peptide	Protein MASCOT score	(SC %)	Total number of peptides	Number of unique peptides	Number of spectra for unique peptide	Protein MASCOT score	(SC %)	Theoretical unique peptides
CYP 1a2	P00186	58.1	9.6	2	2	1	106.2	5.5	ND	ND	ND	ND	ND	27
CYP 2c70	Q91W64	56	9.1	1	1	5	47.3	2.2	2	2	3	40.6	4.9	26
CYP 2d9	P11714	56.9	5.9	3	2	6	95.3	5.4	4	2	4	184.5	8.9	14
CYP 2d10	P24456	57.2	6.2	5	3	7	160.8	11.1	6	2	4	241.8	12.9	12
CYP 2d26	Q8CIM7	56.9	6.2	4	3	2	137	10.6	ND	ND	ND	ND	ND	21
CYP 2e1	Q05421	56.8	9.3	4	3	3	83.4	11.6	3	3	2	83.2	6.3	27
CYP 2f2	P33267	55.9	8.8	1	1	1	35.2	2.4	ND	ND	ND	ND	ND	25

M.Wt; molecular weight, SC%; percent of sequence coverage.

### **3.3.2.3 Quantitative analysis of Cytochrome P450 enzymes extracted by OG and SCX**

The four methods were compared by calculating the CYP450 iTRAQ ratios of three extraction methods (CP, CP+S, and D) relative to disperse homogenization (H). Two criteria were applied to determine the significantly altered CYP isoform as discussed previously in Section 2.2.1.12. The results indicated that the seven CYP450 enzymes identified by OG were within the normal range among the four extraction methods. On the other hand, CP method in SCX showed one CYP450 isoform (CYP2d9) below the normal range while the other three CYP450 isoforms (CYP2c70, 2d10m, and 2e1) were within the normal range (Table 3.4). Although not statistically significant, the p-value for the CP method from the OG data was also low, suggesting that this protein was specifically affected by the extraction method, relative to the H method.

**Table 3-4. Identified cytochrome P450 isoforms by OG and SCX methods, with their mean iTRAQ ratios generated from unique peptides. \***  
indicated the p-value for each method which has been computed following permutation test. ND indicated; not detected

	<b>Separation method</b>	<b>OG</b>			<b>SCX</b>		
CYP isoforms	Extraction method	Mean iTRAQ ratios	Number of unique peptides	p-value*	Mean iTRAQ ratios	Number of unique peptides	p-value*
<b>CYP 1a2</b>	CP/H	0.82	3	0.31	ND	ND	ND
	CP+S/H	1.01		0.99	ND		ND
	D/H	0.97		0.86	ND		ND
<b>CYP 2c70</b>	CP/H	0.95	5	0.76	0.73	4	0.11
	CP+S/H	1.08		0.68	0.79		0.22
	D/H	0.90		0.53	0.86		0.40
<b>CYP 2d9</b>	CP/H	0.75	7	0.05	0.60	6	0.01
	CP+S/H	0.80		0.19	0.73		0.06
	D/H	0.94		0.64	0.83		0.23
<b>CYP 2d10</b>	CP/H	0.94	9	0.61	1.01	8	0.95
	CP+S/H	1.02		0.90	1.18		0.26
	D/H	1.10		0.45	1.19		0.21
<b>CYP 2d26</b>	CP/H	0.84	5	0.27	ND	ND	ND
	CP+S/H	1.00		0.98	ND		ND
	D/H	0.87		0.37	ND		ND
<b>CYP 2e1</b>	CP/H	0.91	7	0.52	0.94	5	0.72
	CP+S/H	0.95		0.75	1.05		0.764
	D/H	1.07		0.66	0.74		0.08
<b>CYP 2f2</b>	CP/H	0.75	2	0.23	ND	ND	ND
	CP+S/H	0.73		0.24	ND		ND
	D/H	0.73		0.19	ND		ND

### 3.4 Discussion

This chapter aimed to identify the best method for protein extraction from fresh and frozen mouse liver samples using four mechanical methods and to investigate the resolving power of two well-established peptide separation methods. The effect of freezing on total proteins content was evaluated as well. Several studies investigated the influence of freezing on protein stability from various organs such as liver from human, rats, and mice (Zeisler et al., 1988, Prentø, 1997, Shabihkhani et al., 2014). Overall findings were not consistent and this may be because of variation in methods of freezing and temperature. However, global thoughts tend to propose that protein freezing, especially for the long duration of time, reduce total proteins content due to formation ice crystals inside the cells (Shabihkhani et al., 2014). For instance, an earlier study proposed that using a fresh sample is the best for subcellular proteomics analysis, as they recovered 13.5% and 14.6% more proteins from fresh liver compared to freshly froze liver or previously frozen liver, respectively (Song et al., 2006). Correspondingly, our results are to some extent in agreement with this finding, where (CP; 7.2%, CP+S; 9.7%, H; 5%, and D; 12.7%) marginally higher protein-to-tissue ratios were detected in fresh liver compared to frozen by the four extraction methods.

The ultimate goal of protein extraction is to maximise the percentage of the recovery of proteins from any finite biological samples as proteins cannot be amplified artificially as in the case of DNA. An additional aim is to separate proteins from other cellular biomolecules, and thus can be detected and investigated in downstream analytical methods, such as 1D or 2D-PAGE,

Enzyme-linked immunosorbent assay (ELISA), Western blotting, MS, or other. In order to detect as wide a range of proteins as possible, optimal protein extraction must be determined experimentally depending on the nature of the sample and stability of proteins (Grabski, 2009). Thus, four mechanical extraction methods (P, CP+S, H, and D) were employed as a single-step or two-step protein extraction methods. Whereby, CP+S method gave a significantly higher yield using fresh and frozen samples of proteins compared to single methods (CP, H or D).

Different approaches have been optimized for extraction and purification of total or specific group proteins from the liver. An earlier study investigated the toxic effect of acetaminophen on mice livers by 2D-GE approach homogenised mouse liver in lysis buffer using handheld homogenizer followed by sonication for a short while (30 seconds) (Albertini and Suter, 2000). The study proposed a significant improvement in the number and confident of protein identifications as result of combining these two-step extraction methods compared to a single-step method (i.e. sonication). Another study homogenized mouse liver by Dounce homogenizer (Song et al., 2006). According to the results, the study demonstrated 0.142 mg protein to mg tissue compared to 0.158 mg protein to mg tissue determined by our D method in this project. Moreover, authors showed organelles such as nuclei and vesicles were integrated (unbroken), proposing the importance of using advanced extraction method or two-step extraction method rather than using mild-method (i.e. Dounce) in liver protein extraction (Song et al., 2006). Moreover, a newly developed machine based on cycling pressure (Pressure-cycling technology - PCT, MicroPestle) was evaluated and compared to PCT-MicroCap by Shao *et al*, (Shao et al., 2016).



Mouse organs including liver with different weights were employed in this evaluation. A total of 0.11 mg protein to mg liver tissue was determined by PCT- MicroPestle. Comparing to our result, CP+S identified double ratio as PCT- MicroPestle determined (0.247 mg protein to mg tissue, fresh liver).

In conclusion, cryo-pulverisation is a non-heating production method. Therefore, it is considered as a valuable technique for active enzyme extraction (Burden, 2008), although it was not the best single-step method for protein extraction. The combination of cryo-pulverization followed by sonication – CP+S is an efficient mechanical method that has been created and evaluated for the first time in liver protein extraction. A previous attempt on lactate dehydrogenase (LDH) enzyme extraction from muscle by various single-step and combined-step approaches, proved that combining cryo-pulverization step with another step such as sonication resulted in the highest activity of LDH, thus highest amount. While sonication as a single step has shown the lowest activity, indicating poor efficiency as a single-step (Burden, 2012b).

OFF-GEL isoelectric focusing (OG) or Strong Cation Exchange chromatography (SCX)-based shotgun proteomics approaches were compared to determine which would be best suited for protein identifications for mouse tissues but in particular liver samples.

Although whole liver extract (rather than microsomes) was used for this comparison, OG was found superior to SCX in identifying more proteins and enriching the number of hydrophobic “membrane” proteins (Figures 3.11-12).

Various studies were performed to evaluate the power of OG method over other separation methods like SCX. A previous attempt by Slebos and his group investigated whether OG (narrow pH, 3.5-4.5, 24 cm) or SCX was better for 10 or 100 µg of protein extract from human colon adenocarcinoma (RKO) cell or from 50 µg of protein from rectal adenocarcinoma biopsy specimen (Slebos et al., 2008). In contrast to our findings, the results determined that SCX generated more peptides and proteins using 10 and 50 µg compared to OG, while equal numbers of identifications were observed using 100 µg of protein. Though, Slebos *et al* employed a narrow range IGP strip in order to reduce bias toward highly redundant identifications of abundant proteins which excluded many peptides and subsequently proteins whereas SCX encompasses all peptides (Cargile et al., 2005). However, with 50 µg, they found that 77% of proteins identified using OG or SCX were common, which was similar to our observations of 68.2% common identities (Slebos et al., 2008). In the same way, Mostovenko and his colleagues found that SCX (200 µg) demonstrated better protein and peptide yields from *Escherichia coli* and human plasma extracts than OG (100 µg) even with wide range IPG strip (pH, 3-10) (Mostovenko et al., 2013).

On the other hand, Slebos *et al* concluded that OG offered superior reproducibility and resolution for peptides separation from large (100 µg) and small (10 µg) protein samples, even though more peptides and proteins were identified by SCX and this was because three replicates were required for detection of 90% of medium abundance proteins (those detected with at least 3-4 peptides) by OG. In contrast, the SCX required six replicates to detect the 90% of medium abundance proteins (Slebos et al., 2008).

Furthermore, protein samples derived from testis of rat (*Rattus norvegicus*) demonstrated that OG, narrow pH range (3.5-4.5) was superior method in proteins separation comparing to SCX method using 1 mg of sample, since it produced 13% more identifications of protein than an optimized off-line SCX (Essader et al., 2005). Moreover, Schafer *et al* have evaluated the separation power of OG and SCX using mouse liver, in order to improve the sensitivity of selected reaction monitoring-based (SRM) quantitative proteomics workflows (Schäfer et al., 2012). They showed that applying separation of peptides by OG using a wide range of IPG (pH 3-10) was more efficient than SCX in increasing the number of quantifiable peptides and signal intensity as well as signal-to-noise ratios. Besides, OG was able to identify six membrane proteins with high signal intensity. In another experiment done by Elschenbroich and co-workers (Elschenbroich et al., 2009) to test the power of the OG method for membrane proteins recovery, using a membrane-enriched fraction from murine C2C12 myoblasts, they found that OG (12 fractions, pH 3-10) and SCX were effective, where the former showed a slight bias toward membrane proteins identification.

Other experiments comparing OG and SCX (Bandhakavi et al., 2009, Tran et al., 2009, Waller et al., 2008, Barnea et al., 2005) were less clear, most likely due to inconsistent experimental conditions such as, sample complexity, starting amount, number of fraction produced, IPG strip length and pH range. However, inclusion of the OG method into shotgun proteomic workflow offers a number of advantages include: (i) determination of the pI of the peptides, which is a unique feature that is not provided by SCX and RP, (ii) loading capacity (up to 50 mg, compared to 250 µg for SCX). (iii) removal of detergents

and denaturants, if they have been used as part of the protein extraction protocol (iv) different pH range IPG strips are available for experimental design flexibility (Hörth et al., 2006). On the other hand, the main disadvantage of the OG is the limitation in selecting the number of fractions, besides, the predefined size and shape of wells. Moreover, OG focusing run time can be long dependent on the sample composition, taking up to 2–3 days.

None of the previous comparative studies evaluate the separation power of OG and SCX quantitatively using liver in a comprehensive proteomics experiment. In this work, the digested peptides were labelled with (iTRAQ) to enable quantification. CYP450 family is highly expressed in liver and it is a good example of membranous bounded proteins, therefore CYP450 isoforms were compared amongst the four extraction methods and the two separation methods. OG detected almost the double of CYP450 enzymes observed with SCX. The 7 CYP450 enzymes identified by OG were within normal range among CP, CP+S, and D extraction methods. On the other hand, in SCX, only one CYP 2d9 was marginally decreased by CP. The number of those identified CYP450 isoforms will be evaluated intensively and compared to literature publications in next chapter (Chapter 4)

In conclusion, pharmacoproteomics studies require a high resolution and reproducible first dimension separation method and OG proved to be the most appropriate for further application in the project.

## Chapter four: Quantitative pharmacoproteomics for investigating the effect of major anti-cancer drugs on mouse liver

## **4 Quantitative pharmacoproteomics for investigating the effect of major anti-cancer drugs on mouse liver**

### **4.1 Introduction**

Drug toxicity (resulting in serious health problems) is one of the major complications leading to the withdrawal of approved drugs from the market (Hay et al., 2014). The liver is able to give accurate and sensitive indications of drugs induced-toxicity. Although, the pronounced advancements in proteins fractionation tools, such as; multidimensional proteomics (i.e. shotgun proteomics) reduce liver protein complexity and thus, identifying low abundance proteins, other approaches have been developed to enrich a set of interest proteins, such as those existing in specific intracellular compartments (Huber et al., 2003), biological complexes (Ho et al., 2002), or those modified proteins (e.g. phosphorylation, glycosylation, etc.). (Oda et al., 2001).

In subcellular fractionation, animal cells are divided into subcellular compartments named “organelles” include: mitochondrion, lysosome, phagosome, ER, Golgi, nucleus, nucleolus, and many others (Jung et al., 2000). Each organelle comprises a characteristic set of proteins that are required to perform particular organelle functions. Some organelles can be isolated to enable understanding of the proteins present and how they enable the organelle to function (Peng et al., 2010).

Liver microsomes are an example of an organelle derived from the ER, which is enriched with a myriad of membranous (e.g. CYP450s) and non-

membranous proteins (e.g. NADH-ubiquinone oxidoreductase chain 1-5) (Golizeh et al., 2015). Liver microsomal proteins have major roles in the biosynthesis of lipid and cholesterol, formation of hormones and drug metabolism (Cederbaum, 2015). Therefore, liver microsomes provide an in-depth insight into xenobiotics metabolism, lipid-protein interactions, the role of membranous enzymes, and drug-drug interactions. Furthermore, the synergistic effect of using liver microsomes with the new advanced techniques of proteins separation have extended the application of shotgun proteomics in the analysis of membranous proteins and helped in overcoming some of the challenges in studying these types of proteins.

The main purpose of traditional anti-cancer agents is to stop the extensive cell division by interfering with their replication at one or more checkpoints in their cell cycle, however, this may result in serious side effects (Weinberg, 2013).

Considering the heterogeneous properties of cancer, most subtypes of cancer are treated with combined-chemotherapeutics agents. An example of chemotherapy combination in treating advanced colorectal cancer is 5-fluorouracil (5FU) with irinotecan or oxaliplatin which enhanced the response rates to 40–50% instead of 10-15% by 5FU alone (Kuehr et al., 2004, Zhang et al., 2008). However, this combination creates additional stress on the liver, as at least one CYP450 isoform (Table 4.1) metabolises most of the anti-cancer drugs (ACDs). Generally, adverse side effects of ACDs occur since CYP450 enzymes have limited capacity and their level can be altered (induced or suppressed) because of the administration of concomitant drugs. For example, CYP3A4 plays a key role in metabolizing several ACDs (such as taxanes, vinca-alkaloids and new drugs such as imatinib, sorafenib, and

gefitinib), therefore, administering a potent CYP3A4 inhibitor such as, ketoconazole will lead to a decrease in the clearance rate of these ACDs with a subsequent increase in their levels in the body (Akhdar et al., 2012). The reduction in clearance of concomitant ACD, which may reach 49% as in the case of docetaxel, would result in a higher toxicity outcome of the drug (Engels et al., 2004b). Similarly, the low expression of CYP3A4 in breast tumour tissue potentiates the response to CYP3A4-metabolized ACDs (Miyoshi et al., 2005).

In addition, CYP450 enzymes are responsible for bioactivation of prodrugs into therapeutic active forms, such as cyclophosphamide, ifosfamide, dacarbazine, procarbazine, tegafur, thiotepa and 1,4-bis-([2-(dimethylamino-N-oxide)ethyl]amino) 5,8-dihydroxy anthracene-9,10-dione (AQ4N) (Rodriguez-Antona and Ingelman-Sundberg, 2006, Nishida et al., 2010). AQ4N is a bio-reductive prodrug which can be activated by CYP2S1 and CYP2W1 in tumour tissues into a topoisomerase II inhibitor (Nishida et al., 2010).



**Table 4-1. Major anti-cancer drugs with their mediated human metabolizing enzymes.**

Anti-cancer agent	Cytochrome P450 isoform (CYP)	References
<b>Cisplatin</b>	2E1, 3A4	(Mašek et al., 2009)
<b>Cyclophosphamide</b>	2B6, 2C9, 3A4	(Boddy and Yule, 2000, Huang et al., 2000)
<b>Docetaxel</b>	1B1, 3A4, 3A5	(Engels et al., 2004a, Shou et al., 1998)
<b>Doxorubicin</b>	2D6, 3A4	(Kivisto et al., 1995)
<b>Etoposide</b>	1A2, 2E1, 3A4, 3A5	(Zhuo et al., 2004)
<b>Irinotecan</b>	3A4, 3A5	(Santos et al., 2000)
<b>Paclitaxel</b>	2C8, 3A4, 3A5	(Shou et al., 1998, Monsarrat et al., 1998)
<b>Procarbazine</b>	1A1, 2B6	(Rodriguez-Antona and Ingelman-Sundberg, 2006)
<b>Tamoxifen</b>	1A1, 1A2, 1B1, 2B6, 2C9, 2C19, 2D6, 2E1, 3A4, 3A5	(Desta et al., 2004, Crewe et al., 1997)
<b>Topotecan</b>	3A4	(Bai et al., 2003)
<b>Vincristine</b>	3A4	(Zhou et al., 1993)
<b>Ifosfamide</b>	2A6, 2B1, 2B6, 2C9, 2C18, 2C19, 3A4, 3A5	(Huang et al., 2000)

There are only a few published papers on pharmacoproteomics of ACDs, particularly on the liver proteome (Bryan, 2006, Cho et al., 2012b, van Swelm et al., 2013). However, identification of liver toxicity induced by chemotherapy agents is often difficult for the reasons of cross diagnosis with other liver disease symptoms.

A few hepatocyte organelles can be involved in liver toxicity in response to ACDs, such as ER and mitochondria. ER is a dynamic intracellular organelle that plays a central role in the synthesis of all secreted proteins from cells, it also has a role in oxidative responsiveness (Coe and Michalak, 2009, Halperin et al., 2014). Disruption of ER homeostasis may lead to accumulation of unfolded proteins in the ER lumen, a condition referred to ER stress (Foufelle

and Fromenty, 2016). Mitochondria are well-recognized organelles that take part in a variety of cellular metabolism, ATP production and macromolecule biosynthesis (Weinberg and Chandel, 2015). The mitochondria have been attributed to produce abundant amounts of reactive oxygen species (ROS) that promote DNA damage and genetic instability (Murphy, 2009).

Drug-induced hepatotoxicity can manifest by several processes including, necrosis, cholestasis, and steatosis. Generated reactive metabolites may cause excessive damage to mitochondria that can eventually result in cellular necrosis (Nelson, 1995). In other circumstances, impairment of bile-acid transportation proteins by ACDs and/or their metabolites leads to cholestasis (i.e. accumulation of bile salt) that trigger secondary injury to hepatocytes (Pauli-Magnus and Meier, 2006). Steatosis originates by a disturbed fatty acid metabolism through, for example, hepatic  $\beta$ -oxidation pathway; this interruption may lead to intracellular accumulation of small lipid vesicles (Fromenty et al., 1990).

This project has focused on studying the effect of major anti-cancer drugs from different classes, including 5-fluorouracil (5FU), doxorubicin (DOX), cisplatin (CIS) and paclitaxel (PAX) on mouse liver, as represented by protein expression changes in the microsomes.

5-Fluorouracil (5FU) is a drug that belongs to the fluoropyrimidines class and is used commonly for the treatment of many advanced solid types of tumour, such as; colon, rectal, breast, gastric, pancreatic, ovarian, bladder and liver cancer (Longley et al., 2003). 5FU has anti-metabolite activity by inhibiting thymidylate synthase (TS) enzyme, which later blocks the formation of

thymidylate, an essential precursor of DNA synthesis and replication (Longley et al., 2003).

5FU has been shown to exhibit hepatotoxic effects, such as increased serum level of aminotransferases, lactate dehydrogenase and alkaline phosphatase, signifying liver injury (Ray et al., 2007). Several reports have demonstrated the hepatotoxic effect of 5FU since it is metabolised extensively in the liver (more than 80%) producing a toxic intermediates fluoro-beta-alanine (FBAL), beta-ureidopropionase (UPB1), etc. (Al-Asmari et al., 2016a, Conklin, 2004, Al-Asmari et al., 2016b, Ray et al., 2007, Ali, 2012). 5FU treatment results in accumulation of fat globules in the hepatocytes (hepatic steatosis) (Miyake et al., 2005). Subsequently, disrupts lipid metabolism via the  $\beta$ -oxidation pathway (Zorzi et al., 2007, McWhirter et al., 2013). However, none of the studies systemically investigated the influence of 5FU on liver. (Kaplowitz and DeLeve, 2013). Although the capability of 5FU to modulate liver CYP has been investigated, the mechanism remains unclear (Stupans et al., 1995, Baumhäkel et al., 2001). Afsar and his co-workers have revealed that 5FU modulated the activities of CYP450 enzymes (CYP2c11 and CYP3a sub-family members) in male rat liver. Furthermore, they elucidated that these alterations could be due to the changes at synthesis level (Afsar et al., 1996). Moreover, several case reports have demonstrated the interaction between 5FU and co-administered drugs; warfarin and phenytoin. In this context, authors suggested that 5FU modulates the synthesis of CYP450s (Saif, 2005, Brickell et al., 2003, Aki et al., 2000). For instance, Aki *et al* suggested that 5FU modulates the expression of CYP2C9, which is thought to be the key isoform of human liver participating in warfarin metabolism (Daly and King,

2003). Further, similar interaction has been observed with a 5FU prodrug, capecitabine which inhibits CYP2C9 synthesis, resulting in elevated warfarin activity (Copur et al., 2001, Yildirim et al., 2006). Conversely, an attempt by Park *et al* used human liver microsomes to investigate the inhibitory effect of 5FU on 13 different CYP450s, including CYP2C9. By using drugs as substrates (e.g. phenacetin O-de-ethylation by CYP1A2, warfarin metabolism by CYP2C9, paclitaxel 6 $\alpha$ -hydroxylation by CYP2C8, and midazolam 1-hydroxylation by CYP3A4), they suggested that there was little or no impact (Park and Kim, 2003).

Doxorubicin (DOX) is a member of the anthracycline antibiotics which induces cytotoxicity by; i) intercalating between DNA base pairs forming “doxorubicin-DNA adducts” which inhibit the progression of the enzyme topoisomerase II, thus blocking the process of replication, ii) generation of free oxygen species, and iii) lipid peroxidation as well as binding to membrane lipids (Hammer et al., 2010, Yang et al., 2014). The end results of DOX administration are DNA synthesis inhibition, apoptotic activation, and death of rapidly dividing cells (Yang et al., 2014). DOX is a highly effective anti-neoplastic agent, used to treat several adult and paediatric tumours such as bladder, breast, lung, ovarian, stomach, thyroid cancers (Granados-Principal et al., 2010). Although DOX is an effective agent, it has shown serious side effects primarily on the heart as well as other organs such as liver, kidney, and brain (Tacar et al., 2013). However, due to rigorous metabolism in the liver, several *in vivo* and *in vitro* studies on DOX reported hepatotoxicity induced by DOX (Wang et al., 2009, Lee et al., 2002, Barraud et al., 2005, El-Sayyad et al., 2009, Lai et al., 2009). DOX exhibits severe changes at histological, structural and

biochemical levels in tested liver, indicating DOX-induced hepatotoxicity (El-Sayyad et al., 2009). The hepatotoxicity can manifest as necrosis, steatosis, fibrosis, cholestasis, and vascular injury (Ishak and Zimmerman, 1995). It is thought to result from DOX inducing oxidative stress, which in turn is responsible for mitochondrial dysfunction and apoptosis (Damodar et al., 2014, Bulucu et al., 2009). Additionally, DOX induces inflammatory changes as measured by C-reactive protein and fibrinogen in the liver, as well as heart and kidney tissues of DOX-administered rats (Deepa and Varalakshmi, 2005).

Few studies have reported the modulation of hepatic CYP450 enzymes by DOX administration. Zordeky *et al* investigated the ability of acute DOX toxicity in modulating the expression of different CYP450 enzymes using rat liver microsomes at both gene and protein expression levels. The results demonstrated that DOX altered the expression of four isoforms (CYP1B1, 2B1, 2C11, and 4A). In addition, the study showed up-regulation in inflammatory mediators; interleukin 6 (IL-6), inducible nitric oxide synthase (iNOS), and tumour necrosis factor – (TNF $\alpha$ ), proposing them as indicators of liver damage (Zordoky et al., 2011).

Cisplatin (CIS) is a powerful anti-neoplastic drug belonging to alkylating agents group. CIS shows clinical activity against a wide variety of solid tumours such as the bladder, ovarian, head and neck, and cervical (Siddik, 2003). The mechanism of action is based on the formation of inter- or intra-strand cross-links called adducts, causing an alteration in the structure and/or function of DNA and subsequent long-term damage for DNA (Siddik, 2003).

A recent study demonstrated that induction of the expression of unfolded response protein (URP) in response to ER stress is a possible mechanism of CIS-induced apoptosis (Mandic et al., 2003). Accordingly, and similar to 5FU, CIS caused hepatic steatosis, suggesting it to interfere mitochondrial and ER functions (Sharma et al., 2014). Hepatotoxicity of CIS recently became well characterised, where production of oxidative stress and depletion in glutathione levels are thought to play a pivotal role as one of the mechanisms (Lu and Cederbaum, 2007). Therefore, several studies have paid attention to identifying and analysing new compounds capable of protecting the liver from these reactive species, such as vitamin E and selenium (Nazıroğlu et al., 2004). A recent proteomics study revealed that CIS modulated the expression of three CYP450s (CYP2c13, CYP2d1, and CYP2d5) at the protein level and four CYP450s (CYP2c12, CYP2e1, CYP4a1, and CYP26b1) at the gene level in primary rat hepatocytes (Cho et al., 2012a). A further study investigated the platinum derivative, satraplatin (JM216), in modulating CYP450s using human microsomes, which indicated a strong but non-specific inhibitory effect (Ando et al., 1998).

Paclitaxel (PAX) is a mitotic inhibitor originally derived from the bark of the Pacific yew tree, *Taxus brevifolia* (Stierle et al., 1993). PAX is commonly used in several types of solid cancer, however, it is the first line choice for advanced ovarian, non-small cell lung cancers and metastatic breast cancer (Khanna et al., 2015). Contrary to the microtubule destabilizing agents (e.g. vinca alkaloids), the primary mechanism of action of PAX is the prevention of cytoskeletal microtubule disassembly through its direct binding to microtubulin, resulting in suppression of cell mitosis and induction of apoptosis (Khanna et

al., 2015, Walsh and Goodman, 2002). Similar to previous mentioned ACDs, PAX has shown significant degenerative and necrotic effects on mice liver tissues (Karaduman et al., 2010). PAX is extensively metabolised in the liver via CYP2C8 and CYP3A4; however, there is no evidence indicating that PAX modulated the expression of liver CYP450s.

The compound 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) is a well-established CYP450 enzymes inducing agent, it has been used as a control for CYP450s for both qualitative and quantitative analysis of liver microsomes in the animal model (Smith et al., 1993). The mechanism of inducing CYP450 genes is due to the activation of TCPOBOP for the constitutive androstane receptor (CAR). CAR plays a role in hepatic detoxification pathways in response to xenobiotic or endogenous stimuli (Baskin-Bey et al., 2006). Although TCPOBOP is a CYP450 inducer, Lane *et al.*, have observed down-regulation of four CYP isoforms (CYP2C40, 2E1, 3A41, and 27A1) prior to a single dose of TCPOBOP administered (Lane et al., 2007).

Traditional methods for CYP450 enzymes identification have been based on implicated specific inhibitors or substrates (Kobayashi et al., 2003), antibody-based identifications (Shou et al., 2000) or measuring the corresponding mRNA level (Patterson and Murray, 2002). The disadvantages of traditional methods can be summarised by the lack of specificity and sensitivity due to the high degree of CYP450 enzymes sequence homology that was observed not only within one CYP450 family but also among various disparate isoforms. To overcome the limitations of these methods; highly validated and sensitive strategies were established by taking advantage of MS advancement. Since

all CYP450 molecular weights are located between 48-62 kDa range, SDS-PAGE has been widely used for first dimension microsomal proteins' separation, embracing detergents for microsomal purification prior to LC-MS protein identifications (Lane et al., 2004, Nisar et al., 2004a, Lisitsa et al., 2009, Shrivastava et al., 2013b, Sutton et al., 2010).

Galeva and his colleagues evaluated the efficiency of one and two-dimensional gel electrophoresis (1D-PAGE, 2D-PAGE) for separation of hydrophobic membranous proteins, they demonstrated that 1D-GE is more applicable for CYP450 enzymes identifications than 2D-PAGE due to the aggregation of these proteins during first dimension separation of 2D-PAGE. In addition, 1D-PAGE resulted in identification of 7 CYP450 enzymes in livers from control and phenobarbital-treated mice (CYP 2a1, 2b1, 2b2, 2c11, 2d2, 2d5 and 3a1), whereas 2D-PAGE identified only 2 isoforms (CYP2b1 and 2b2) (Galeva and Altermann, 2002). Applying in-gel digestion approach for CYP450s identification has been reported firstly by Nisar and co-workers (Nisar et al., 2004b). Using rat liver microsomes, they positively identified 24 CYP450 isoforms. Another attempt by Sutton *et al* using an enhanced 1D-PAGE approach for microsomal proteins separation identified 26 of highly homologous CYP450 isoforms from mouse (Sutton et al., 2010). A major drawback of using gel-based approach is the limited number of families that can be identified, low sensitivity, and lack of quantification. Conversely, gel-free proteomics approach is commonly used nowadays for profiling the whole proteome, which raises a challenge to identify a high number of microsomal proteins including CYP450 enzymes. Due to the use of optimised proteomics



workflow (Chapter 3), a remarkable number of CYP450 isoforms were significantly identified and quantified in this Chapter.

#### **4.1.1 Aims of the chapter**

Work in this chapter has been conducted in two independent quantitative comparative experiments with the main aims to:

- a. Investigate the effect 5-flourouracil (5FU), doxorubicin (DOX), paclitaxel (PAX), cisplatin (CIS), and the CYP450s inducer, 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) on mouse liver microsomes by the means of proteomics technique.
- b. Identify potential biomarkers that may have role in drug disposition and/or liver toxicity
- c. Identify the changes in CYP450 expression, along with other xenobiotic enzymes as result of the application of these drugs.

## **4.2 Material and Method**

### **4.2.1 Reagents and chemicals**

5-fluorouracil (5FU), cisplatin (CIS), doxorubicin (DOX), paclitaxel (PAX), 1,4-Bis [2-(3,5 dichloropyridyloxy)] benzene (TCPOBOP), Cremaphor-EL (CrEL), dimethyl sulphoxide (DMSO) and Arachis oil were purchased from Sigma-Aldrich, UK. For information about other materials used in this chapter, refer to section 2.1.1.

### **4.2.2 Animal treatment and xenograft preparation**

#### **4.2.2.1 Animals**

Male and female BALB\cOLaHsd–Foxn1nu immunodeficient nude mice (Harlan Laboratories, UK) aged 10 weeks and 15 weeks, respectively, were used. All mice were kept and maintained as described previously (section 3.2.1).

#### **4.2.2.2 Cell lines**

Human colon adenocarcinoma (DLD-1) and human pancreatic carcinoma (PANC-1) cell lines were purchased from ECACC (Porton Down, Wiltshire, UK). DLD-1 and PANC-1 were selected due to their good vascularisation when grown *in vivo* as subcutaneous xenograft tumours. Both cell lines were cultured in RPMI-1640 cell culture medium supplemented with 1mM sodium pyruvate, 2mM L-glutamine and 10% fetal bovine serum (Sigma-Aldrich, UK).

#### **4.2.2.3 Tumour system**

Tumours were excised from a donor animal, placed in sterile physiological saline containing antibiotics and cut into small fragments of ~2 mm<sup>3</sup>. Under brief general inhalation anaesthesia, cell line fragments were implanted in both flanks of each mouse using a trocar. Once the tumours could accurately be measured by calipers, the mice were divided into groups of 8 by restricted randomisation in order to minimise group mean tumour size variation.

#### **4.2.2.4 Chemotherapy treatment**

Animal husbandry and tissue collection were carried out by Home Office-certified expert Tricia Cooper, at the Institute of Cancer Therapeutics, University of Bradford. 5FU, CIS, DOX, PAX, and TCPOBOP were used to treat mice in two independent experiments. A colleague of the proteomics' team had prepared the first set (Set A) previously. Male mice for Set A were treated with 5FU, CIS and DOX and female mice for Set B with 5FU, PAX, and TCPOBOP. In each case, Control mice were included receiving no treatment for Set A and normal saline for Set B. 5FU was included in both studies as an inter-experimental control (Table 4.2). Animals were sacrificed 24 hours after treatment. Heart, lung, liver, kidney, and tumour were recovered and stored at -80°C for proteomics analysis. Only the livers were used for subsequent experiments.

Drugs doses (Table 4.2) which were used here were determined based on independent experiments prior to the present project on mice. The dose was measured as the maximum tolerated dose by mouse. Several toxic signs were considered in determining the dose; however, weight loss by maximum 15%

was the main indicator used for toxicity. Alongside the data from these experiments, the toxic dose used in the literature for these drugs, as well as the tolerated toxic effect of solvents were also taken into account.

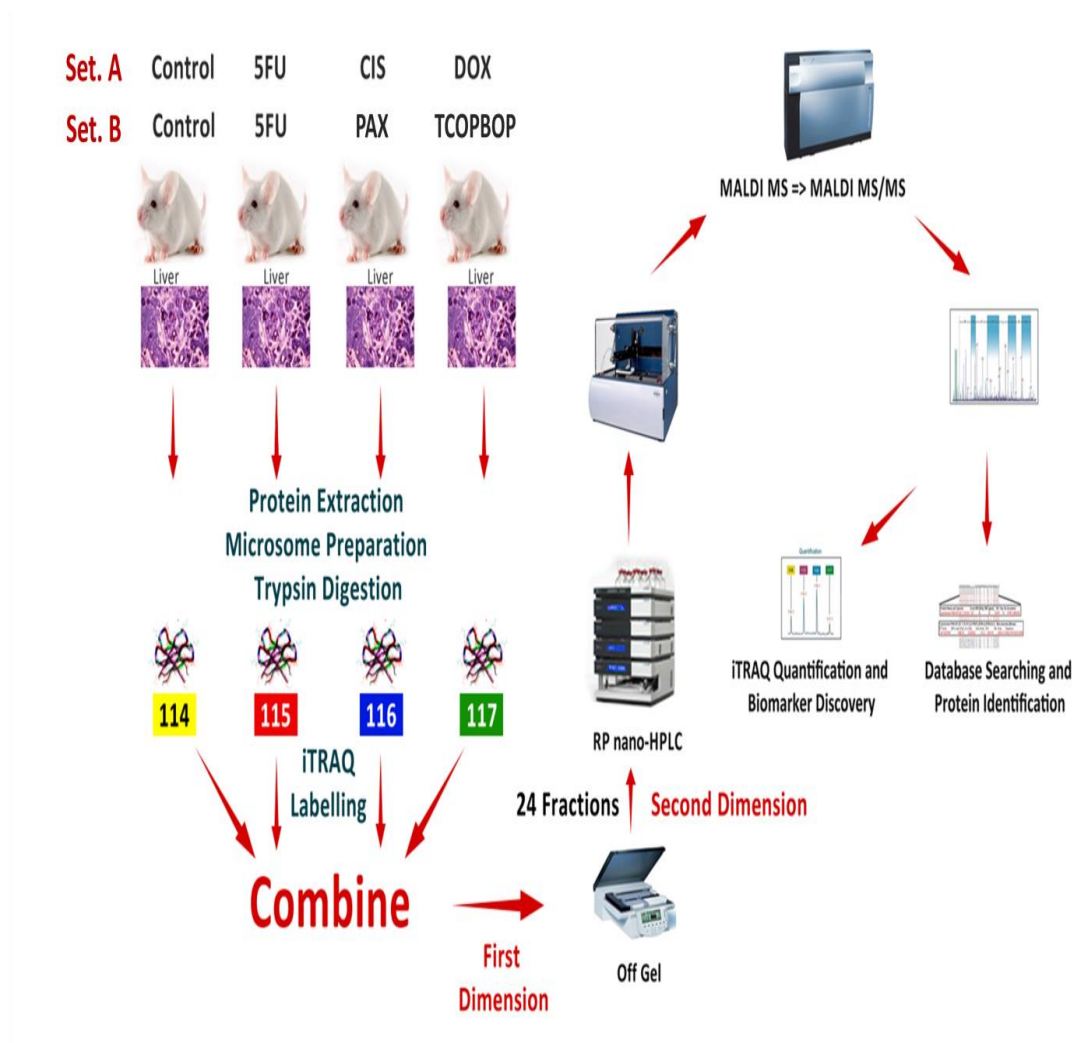
**Table 4-2. Conditions of mice treatment and the type of drugs and tumours used**

Mice set	Mice gender	Drug	Tumour type	Dose (mg/kg)	Route of drug administration	Duration (hr.)	Solvent system
<b>Set A</b>	<b>Male</b>	Control	PANC-1	0	Not given	24	Not given
		5FU	PANC-1	100	IP	24	Normal saline
		CIS	PANC-1	10	IP	24	10% DMSO
		DOX	PANC-1	10	IP	24	Normal saline
<b>Set B</b>	<b>Female</b>	Control	DLD-1	0	IP	24	Normal saline
		5FU	DLD-1	100	IP	24	Normal saline
		PAX	DLD-1	20	IP	24	CrEL, DMSO and Arachis oil
		TCPOBOP	DLD-1	3	IP	24	10% DMSO/Arachis oil

### 4.2.3 Sample preparation

#### 4.2.3.1 Protein extraction from mouse liver by cryo-pulverization and microsomes preparation

The treated mouse livers were subject to quantitative proteomics as illustrated in Figure 4.1. Initially, mouse livers were weighed before dicing into small pieces and then protein extraction by sonication for Set A, where the optimised method (CP+S) was used for Set B as elucidated earlier (sections 3.2.2.1.1-2). Next, the microsomal fraction was prepared for each sample as described in Section 2.2.1.1 in order to enrich drug-metabolizing proteins.



**Figure 4-1. Flow diagram showing the steps of quantitative proteomics workflow that have been used. After drugs treatments, proteins were extracted by dicing followed by sonication method for Set A and the optimised method (CP+S) for Set B. iTRAQ labels were used for proteins quantification. Combined labelled proteins from both sets were subjected to Off-Gel (OG) and RP-HPLC separation techniques. The generated fractions were analysed using MALDI-TOF-TOF-MS for protein identifications by database searching and quantifications.**

#### 4.2.3.2 iTRAQ peptide labelling

Defined amounts (50µg) of each treated sample, calculated by Bradford assay (section 3.2.1.2) were, recovered and digested with acetone and trypsin

enzyme, respectively, (sections 3.2.1.4-6) prior to tagging with iTRAQ 4-plex reagent (section 3.2.1.7), in the two separated sets as shown in Table 4.3.

**Table 4-3. 4-plex iTRAQ reagents was used to label tryptic digests of mouse livers treated with specific anti-cancer drugs or controls.**

<b>iTRAQ reagent</b>	<b>114</b>	<b>115</b>	<b>116</b>	<b>117</b>
	<b>Drug used</b>			
<b>Set A</b>	Control	5FU	CIS	DOX
<b>Set B</b>	Control	5FU	PAX	TCPOBOP

#### **4.2.3.3 Peptide separation and identification**

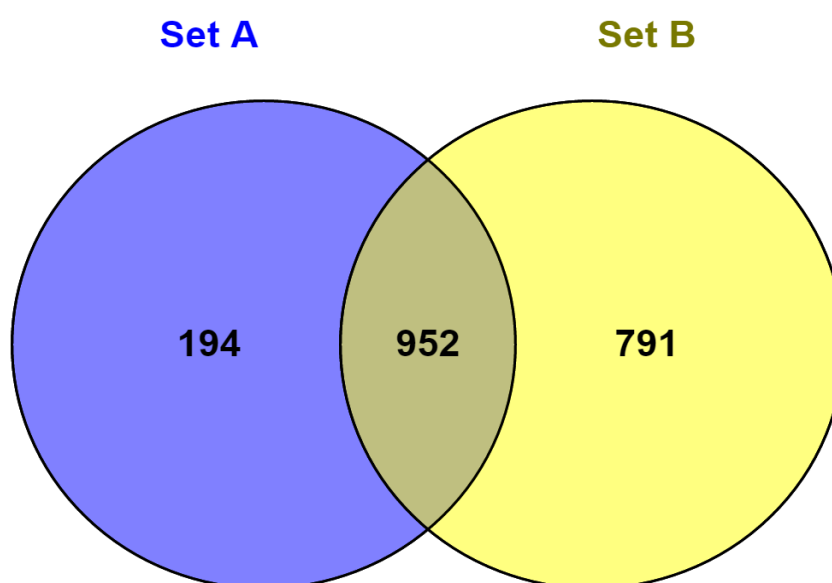
iTRAQ samples for each set were combined into one tube and then separated in two dimensions as shown in Figure 4.1.

The optimised OG separation technique was used for first dimension separation, using a wide pH range IPG strip (3-10, 17cm) and 24 fractions were collected (section 3.2.1.7.1.2). The resulting fractions were desalted as described in Section 3.2.1.5 and separated for the second dimension using RP-HPLC technique (section 3.2.1.7.2). The MS analysis and protein identification were conducted as described in Sections 3.2.1.8-10.

## 4.3 Results

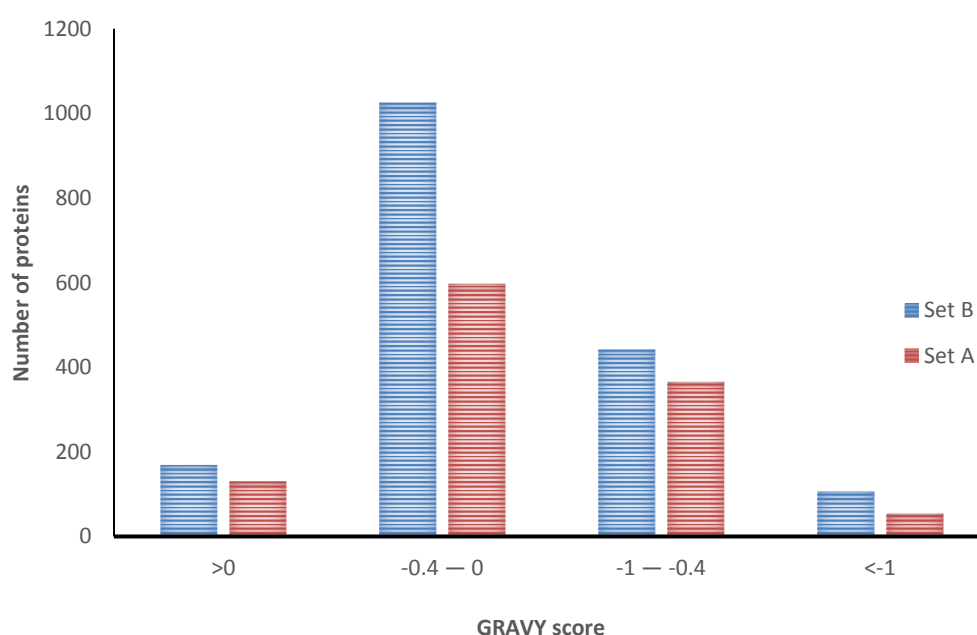
### 4.3.1 Evaluation identified proteins from datasets A and B

Two strategies were employed to extract liver microsomal proteins: a) extraction by dicing and sonication method for Set A (5FU, CIS and DOX); and, b) extraction by cryo-pulverization followed by sonication method for Set B (5FU, PAX, and TCPOBOP). Of collectively identified proteins, a total of 1146 and 1743 proteins were obtained in Set A and Set B, respectively, with good qualitative data (at least two peptides identifying each protein) (Appendix BI and BII). Comparing the identified proteins by datasets A and B revealed that 952 proteins were common between the two sets (Figure 4.2), indicating that Set B identified approximately 83% of those proteins identified by Set A.



**Figure 4-2. Venn diagram showing the number of unique and common proteins between Set A and Set B.**

Next, in order to evaluate the efficiency of the extraction methods of Set A and Set B in recovering microsomal proteins, two approaches were employed. Firstly, the grand average of hydropathy (GRAVY) algorithm (Figure 4.3) was used to determine the hydrophobic status of identified proteins as described earlier in Section 2.2.1.13.



**Figure 4-3. Distribution of identified proteins from Sets A and B over the GRAVY scores. The value (-0.4) is the threshold between cytosolic proteins and membrane proteins, therefore protein with score higher than (-0.4) indicates higher probability for membrane association (a more positive GRAVY score the more hydrophobic a protein)**

GRAVY results demonstrated that 726 proteins (63% of total identified proteins by Set A) and 1194 proteins (69% of total identified proteins by Set B) had GRAVY score of higher than (-0.4), suggesting a high proportion of membrane proteins.



Secondly, the identified proteins in datasets A and B were submitted to Phobius software for prediction of integral membrane proteins to determine protein's hydrophobicity (section 2.2.1.13). Results demonstrated that 32% (368/1146) and 35% (608/1743) from Set A and Set B, respectively were predicted to be transmembrane proteins.

Following this datasets of identified proteins from Set A ( $n = 1146$ ) and Set B ( $n = 1743$ ) were compared based on their cellular component, molecular function, and biological process (Appendix CI, CII, and CIII, respectively) using the Biological Networks Gene Ontology (BinGO) tools for ontology annotation (section 2.2.1.13). In addition, the identified proteins from Set A and Set B were compared to a reference protein list (RPL) provided by BinGO for enrichment and depletion analysis. A hypergeometric test with  $p < 0.01$  as a cutoff was used to determine significant GO terms after correcting for a multiple term testing with a Benjamini and Hochberg false discovery rate. The enrichment factor was computed as the number of dataset proteins and reference proteins (i.e. entire international protein index-IPI of the mouse) annotated to each GO term divided by the number of dataset proteins and entire IPI proteins linked to at least one annotation term within the indicated GO terms.

Analysis based on cellular locations, molecular functions, and biological processes did not show any significant differences between Set A and B. However, cytoplasmic associated proteins (CAPs), which are highly abundant in cells, were presented as the highest abundant fraction among Set A (33.3%) and Set B (25.7%). However, the analysis showed that CAPs were marginally

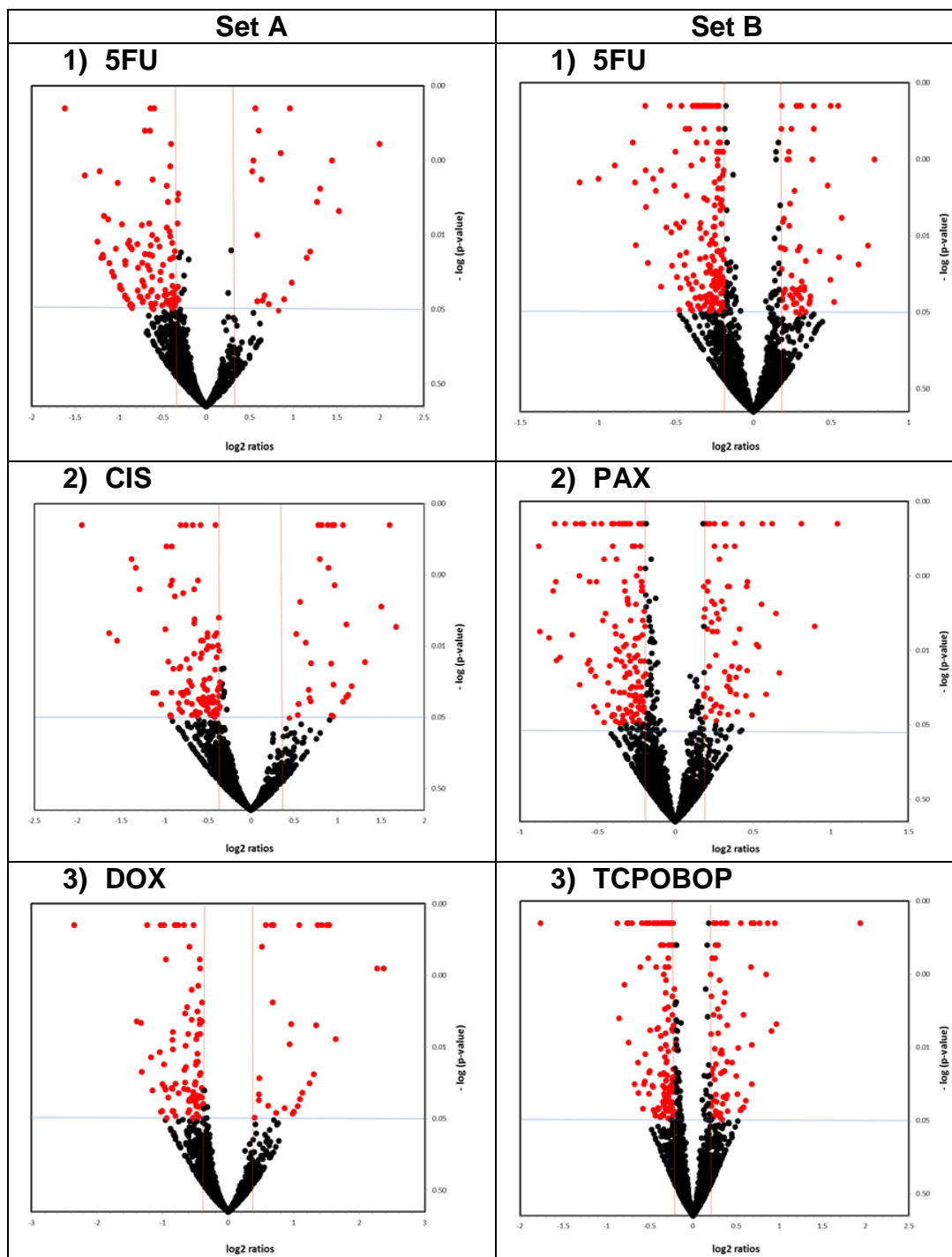
enriched in Set A (p-value, 0.016), and significantly depleted in Set B (p-value,  $5 \times 10^{-8}$ ).

Correspondingly, molecular function analysis of Set B demonstrated significant depletion in proteins involved in, 1) nucleotide, chromatin and DNA binding, 2) receptor binding, and 3) actin binding, indicating a reduction in nuclear, cytoplasmic and plasma membrane proteins, respectively. On the other side, RNA binding and lipid binding were significantly enriched, confirming more ribosomal proteins identified. Similarly, biological processes revealed that proteins are structurally and functionally linked to ER organelle (i.e. rough endoplasmic reticulum).

### **4.3.2 Validation of differentially expressed proteins**

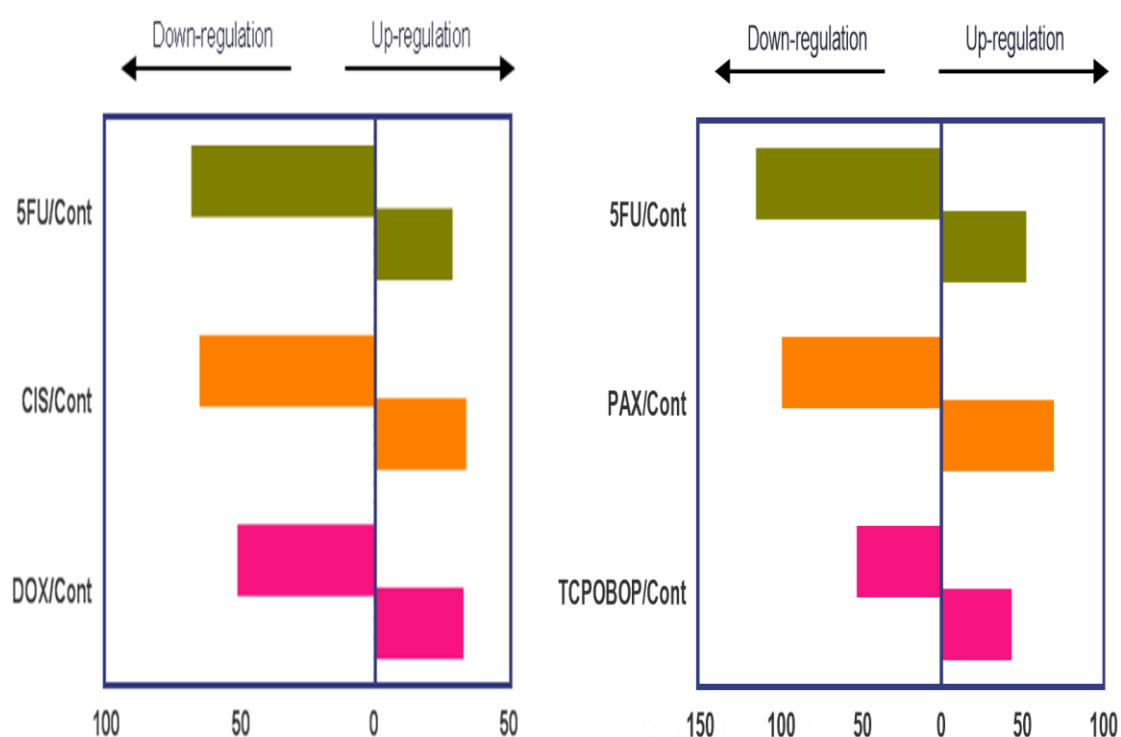
For relative protein quantifications, minimum two unique peptides were required to generate a quantifiable protein list (section 2.2.1.12). Employing this strategy resulted in 1095 (96% of identified proteins) and 1726 (98% of identified proteins) accurately quantified proteins for Sets A and B, respectively. P-value was computed for each protein via permutation test as mentioned previously in Section 3.2.1.10. The distribution of quantified proteins ( $\log_2$ ) against  $-\log$  (p-value) of the ratio is presented using volcano plot for each drug treatment (Figure 4.4).

For significantly altered proteins in each dataset, two criteria were applied as cutoffs for significantly altered proteins. The first cutoff was the protein p-value; thus proteins were considered significantly altered if their (p-value < 0.05). The second cutoff was the standard deviation (SD), proteins were considered as up-regulated if their  $\log_2$  iTRAQ ratios were more than the cutoff (+1SD) and down-regulated if their  $\log_2$  iTRAQ ratios were less than the cutoff (-1SD) (Figure 4.4).



**Figure 4-4. Volcano plot of the complete iTRAQ proteomic dataset for Set A; 5FU, DOX, CIS (left column) and Set B; 5FU, PAX, TCPOBOP (right column) showing the fold change in protein expression between treated and control sample. For significantly altered proteins, 1SD was the first cutoff (the vertical two red dotted lines indicate the up- and down-regulated points). The second cutoff was the p-value (the horizontal blue dotted line indicates the p-value threshold, p-value < 0.05). Proteins were deemed significantly altered under the two criteria were colored with red.**

Of the significantly regulated proteins in Set A, 67, 64, and 50 proteins were down-regulated and 28, 33, and 32 proteins were up-regulated in 5FU, CIS, and DOX, respectively. For Set B, a total of 113, 97, and 51 proteins were down-regulated and 51, 68, and 42 proteins were up-regulated in 5FU, PAX, and TCPOBOP, respectively (Figure 4.5)



**Figure 4-5. Bar charts showing the number of regulated proteins (up- or down-regulated) as result of each drug, (A) for Set A and (B) for Set B.**

### 4.3.3 Functional analysis of significantly regulated proteins

#### 4.3.3.1 Functional analysis of significantly regulated proteins by Set A

The number of modulated proteins by each drug, including the unique and common proteins is shown in Venn diagrams (Figure 4.6).

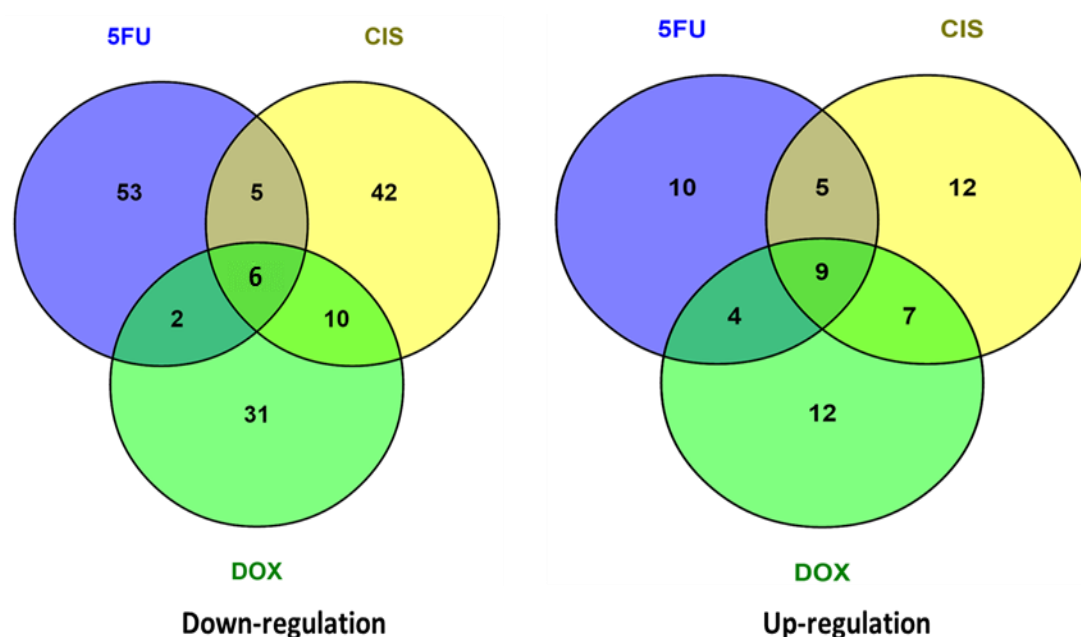


Figure 4-6. Venn diagrams depicting proteins regulated either down or up by 5FU, CIS and DOX-treatment in Set A. Proteins in the blue circle are regulated by 5FU, proteins in the yellow circle are regulated by CIS, and proteins in the green circle are regulated by the DOX treatment.

##### 4.3.3.1.1 Common differentially expressed proteins

Among the differentially expressed proteins in Set A, six and nine proteins were significantly down-regulated (Table 4.4) and up-regulated (Table 4.5), respectively, by the three treatments; 5FU, CIS, and DOX.

**Table 4-4. A list of down-regulated proteins by the three treatments for Set A**

Accessio n	Protein Name	5FU/Cont		CIS/Cont		DOX/Cont	
		iTRAQ ratio	p- value	iTRAQ ratio	p- value	iTRAQ ratio	p- value
P16460	Argininosuccinate synthase	0.73	0.0001	0.65	0.0001	0.63	0.0001
Q9Z2W0	Aspartyl aminopeptidase	0.70	0.0094	0.72	0.0286	0.72	0.0456
Q8CGC7	Bifunctional glutamate/proline--tRNA ligase	0.66	0.0049	0.65	0.0072	0.61	0.0039
P49312	Heterogeneous nuclear ribonucleoprotein A1	0.73	0.0292	0.65	0.0110	0.60	0.0106
P53395	Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial	0.72	0.0063	0.58	0.0009	0.46	0.0001
P12246	Serum amyloid P-component	0.36	0.0001	0.28	0.0001	0.21	0.0001

Most of the down-regulated proteins have an important protective role in response to drugs administration, for example, argininosuccinate synthase (ASS) is an essential rate-limiting enzyme in arginine biosynthetic pathway that catalyses the synthesis of argininosuccinate from citrulline and aspartate. ASS is primarily located in the outer membrane of mitochondria inside the liver, it has been implicated in different types of cancer (e.g. hepatocellular carcinoma) and chemotherapy resistance (i.e. platinum anticancer drugs) (McAlpine et al., 2014a, Delage et al., 2010). Moreover, suppression of ASS showed a significant protection against CILI (Ming Leung et al., 2012). Aspartyl aminopeptidase (ASP) is a cytosolic abundant catalytic enzyme, which has specificity towards acidic peptide/ proteins, most commonly cleaving aspartic acid from the N-terminus and regulating bioactivation of peptides such as angiotensin II. Up-regulation of ASP mRNA level has been linked to the different type of cancer, such as colorectal cancer (Larrinaga et al., 2013).

Heterogeneous nuclear ribonucleoprotein A1; is an mRNA transporter from the nucleus to the cytoplasm. It has shown a significant role in hepatocellular carcinoma progression (Li et al., 2012). Furthermore, it has been considered as a potential target of different anticancer drugs including, DOX (Mandili et al., 2012). Serum amyloid P-component is an apolipoprotein synthesized in the liver and its level increased in response to inflammation (e.g. drug administration) (Villapol et al., 2015). Lipoamide acyltransferase is a component of branched-chain alpha-keto acid dehydrogenase complex (BCKD) which is a mitochondrial enzyme that catalyses the conversion of alpha-keto acids into acyl-CoA. Suppression of BCKD results in accumulation of branched-chain amino acids (i.e. valine, leucine, and isoleucine), which appear capable of reducing oxidative stress effects, suggesting a protective route alongside liver injury (Tretter and Adam-Vizi, 2005). Previous studies demonstrated a reduction in BCKD level in response to the hepatotoxin, tienilic acid and its reactive metabolites (Koen et al., 2012).

The up-regulated proteins are involved in lipid and glycogen metabolism (Table 4.5) including acetoacetyl-CoA synthetase (AACS) which activates acetoacetate to acetoacetyl-CoA that can be further utilized by ketone bodies for fatty acid synthesis (Tisdale, 1984). Up-regulation of AACS was observed in mice fed a high-fat diet as an indication of induction lipogenesis. In drug toxicity induction AACS is an indicator of high levels of free fatty acids (FFAs), thus liver injury (Kirpich et al., 2011). Fatty acid-binding protein (FABP1) is a cytoplasmic protein thought to have a role in binding and transporting lipophilic substrates such as fatty acid, indicating a hepatocellular protective mechanism against free radical products and liver steatosis (Gong et al., 2014).



**Table 4-5. A list of up-regulated proteins by the three treatments for Set A**

Accession	Protein Name	5FU/Cont		CIS/Cont		DOX/Cont	
		iTRAQ ratio	p-value	iTRAQ ratio	p-value	iTRAQ ratio	p-value
Q9D2R0	Acetoacetyl-CoA synthetase	2.45	0.01	2.43	0.02	2.70	0.01
P12710	Fatty acid-binding protein, liver	1.37	0.04	1.56	0.00	1.51	0.07
O35387	HCLS1-associated protein X-1	2.53	0.01	2.10	0.04	3.40	0.00
P11588	Major urinary protein 1	2.18	0.02	2.47	0.02	6.27	0.00
P11589	Major urinary protein 2	1.81	0.01	2.04	0.01	2.78	0.00
P02762	Major urinary protein 6	1.74	0.03	2.21	0.03	3.34	0.00
Q99MR9	Protein phosphatase 1 regulatory subunit 3A	3.17	0.00	3.47	0.00	5.26	0.00
Q8R429	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1)	3.00	0.00	2.07	0.01	1.65	0.04
Q921T2	Torsin-1A-interacting protein 1	2.05	0.04	2.70	0.01	2.18	0.03

HCLS1-associated protein X-1 (HAX1) is a mitochondrial protein, which potentiates cell survival by various pathways such as promoting the clathrin-mediated endocytosis pathway and has been described to be involved in cancer metastasis (Ramsay et al., 2007). HAX1 is up-regulated to counteract the delivery of free fatty acid and liver necrosis, thus promoting hepatocyte survival (Lam et al., 2015). Major urinary proteins (MUP1, 2, 6) are pheromone-binding proteins expressed largely in male mice (Flower, 1996). MUPs are believed to have a cellular protective mechanism and/or drug binding and excretion.

#### **4.3.3.1.2 Unique differentially expressed proteins**

A list of unique differentially expressed proteins in 5FU, CIS, and DOX compared with control are listed in the additional table (Appendix D; DI, DII

and DIII for 5FU, CIS and DOX, respectively). The unique differentially expressed proteins by 5FU, CIS and DOX were compared based on their cellular location and biological process to a reference protein list of complete mouse proteome (IPI mouse) that was provided by BinGO for enrichment study (section 2.2.1.13).

The analysis did not show significant enrichment in regulated proteins by 5FU, CIS or DOX, however, 5FU has exhibited a bias in up-regulation of ER-stress proteins (7 proteins), which would explain the mechanism of 5FU inducing liver injury. CIS showed prominent down-regulation in mitochondrial proteins. This suppression may lead to ROS accumulation, DNA damage, and subsequently hepatocellular death. DOX resulted exclusively in down-regulation of proteins from ER, proteasomes complex and Golgi apparatus. The biological processes related to these particular groups of proteins were involved mainly metabolic energy generation via carbohydrate metabolism. Whilst, up-regulated proteins play an important role in  $\beta$ -oxidation pathway, response to stress and cell division proteins.

#### **4.3.3.2 Functional analysis of significantly regulated proteins by**

##### **Set B**

The number of modulated proteins for each treatment of Set B, including the unique and common proteins, is shown below in Venn diagrams (Figure 4.7).

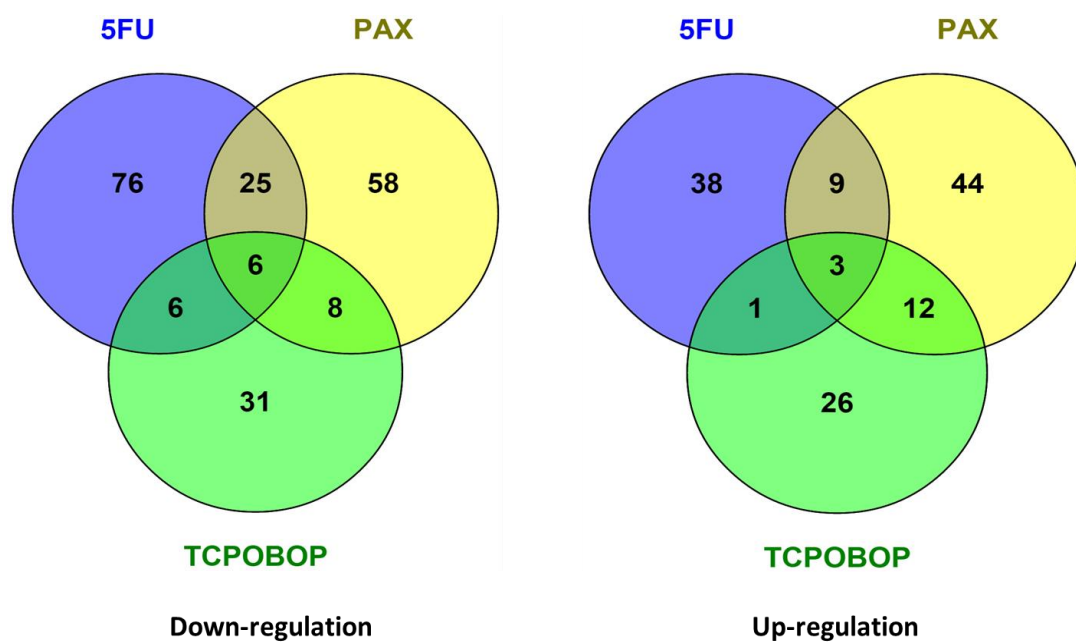


Figure 4-7. Venn diagrams depicting proteins regulated either down or up by 5FU, PAX and TCPOBOP-treatment in Set B. Proteins in the blue circle are regulated by 5FU, proteins in the yellow circle are regulated by PAX, and proteins in the green circle are regulated by the TCPOBOP treatment.

#### 4.3.3.2.1 Common differentially expressed proteins

Among the differentially expressed proteins in Set B, six proteins were down-regulated (Table 4.6) and three proteins were up-regulated (Table 4.7) by all three treatments; 5FU, PAX, and TCPOBOP, respectively.

**Table 4-6. A list of down-regulated proteins by the three treatments for Set B**

Accession	Protein Name	5FU/Cont		CIS/Cont		DOX/Cont	
		iTRAQ ratio	p-value	iTRAQ ratio	p-value	iTRAQ ratio	p-value
Q924Z4	Ceramide synthase 2	0.58	0.00	0.78	0.03	0.70	0.01
P15392	Cytochrome P450 2A4	0.82	0.00	0.71	0.00	0.67	0.00
Q9CR61	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 7	0.77	0.03	0.79	0.04	0.71	0.02
Q7TNG8	Probable D-lactate dehydrogenase, mitochondrial	0.83	0.03	0.76	0.00	0.75	0.00
Q9CQS8	Protein transport protein Sec61 subunit beta	0.54	0.00	0.63	0.00	0.73	0.04
Q61136	Serine/threonine-protein kinase PRP4 homolog	0.74	0.02	0.74	0.01	0.72	0.02

The majority of down-regulated proteins were linked to mitochondrial dysfunction. For example, ceramide synthase 2 is a key enzyme in sphingolipid biosynthesis (Reynolds et al., 2004). It has been reported that depletion in ceramide synthase 2 protein causes mitochondrial dysfunction due to ROS accumulation (Zigdon et al., 2013). CYP2A4 has a role in hydroxylation of several endogenous and exogenous compounds. Drugs causing steatosis such as tetracycline showed down-regulation in the expression of CYP2A4 (Yin et al., 2006). NADH dehydrogenase-1 beta subcomplex subunit 7 (NDUB7) is a member of mitochondrial membrane respiratory chain (complex I) that is responsible for electron transfer. The expression of NDUB7 was down-regulated in non-alcoholic fatty liver disease (NAFLD), suggesting liver injury in our study of ACDs (Wei et al., 2008).

None of the up-regulated proteins have a role in response to drug toxicity or metabolism or liver injury (Table 4.7).

**Table 4-7. A list of up-regulated proteins by the three treatments for Set B**

Accession	Protein Name	5FU/Cont		CIS/Cont		DOX/Cont	
		iTRAQ ratio	p-value	iTRAQ ratio	p-value	iTRAQ ratio	p-value
Q8K1N1	Calcium-independent phospholipase A2-gamma	1.52	0.0034	1.47	0.0053	1.82	0.0006
Q9CPQ3	Mitochondrial import receptor subunit TOM22 homolog	1.72	0.0063	1.63	0.0105	1.90	0.0041
Q9Z2Y8	Proline synthase co-transcribed bacterial homolog protein	1.38	0.0078	1.31	0.0160	1.46	0.0048

#### 4.3.3.2.2 Unique differentially expressed proteins

The unique differentially expressed proteins in 5FU, PAX, and TCPOBOP compared with control are listed in additional tables (Appendix EI, EII, and EIII for 5FU, PAX, and TCPOBOP, respectively). Moreover, the enrichment analysis for unique differentially expressed proteins for each drug (5FU, PAX, and TCPOBOP) was performed as for Set A. The analysis of down-regulated proteins by 5FU showed significant enrichment in ribosomal proteins (14 ribosomal proteins) that have a role in translation, ribosomal assembly, ribosomal subunit biogenesis and protein folding. For up-regulation, mitochondrial proteins were highly enriched, which showed a preference to fatty acid degradation. PAX significantly regulated proteins related mainly to ER-organelle (e.g. CYP450s and carboxylesterase enzyme), which have a major role in various types of metabolic processes such as, oxidation-reduction process, and lipid, fatty acid, ketone, alcohol, and carboxylic acid metabolism. TCPOBOP showed a significant effect on microsomal proteins, especially drugs metabolizing enzymes. TCPOBOP affected bile acid, steroid metabolic process, and fatty acid metabolism. Furthermore, proteins

responding to stress were up-regulated in TCPOBOP, indicating protective role.

#### 4.3.4 Validation of the inter-experimental control (5FU) in quantitative proteomics study

5-Fluorouracil (5FU) was used in both experiments as an inter-experimental control. A total of 925 proteins were confidently identified and quantified in both datasets of 5FU.

Of the commonly identified proteins (925 proteins), 56 and 28 proteins were significantly changed by 5FU in Set A and Set B, respectively. Amongst these proteins, four were down-regulated and seven were up-regulated in both datasets (Table 4.8). The functional analysis of these proteins assigned them to ER and mitochondrion pathways, respectively.

**Table 4-8. The commonly regulated proteins by 5FU in the Sets A and B.**

Regulation	Protein name	Accession	Set A		Set B	
			iTRAQ ratio	p-value	iTRAQ ratio	p-value
Down-regulated in both Datasets	Annexin A6	P14824	0.74	0.0391	0.83	0.0146
	Ferritin heavy chain	P09528	0.71	0.0009	0.82	0.0001
	Protein disulfide-isomerase A3	P27773	0.74	0.0026	0.81	0.0001
	Protein disulfide-isomerase A4	P08003	0.71	0.0411	0.83	0.0001
Up-regulated in both Datasets	3-hydroxyacyl-CoA dehydrogenase type-2	O08756	1.18	0.0489	1.29	0.0209
	Carbamoyl-phosphate synthase [ammonia], mitochondrial	Q8C196	1.4	0.0009	1.27	0.0001
	Glycerol-3-phosphate acyltransferase 1, mitochondrial	Q61586	1.39	0.0478	1.2	0.0146
	Carnitine O-palmitoyltransferase 2, mitochondrial	P52825	1.55	0.0124	1.21	0.0003
	Pyruvate carboxylase, mitochondrial	Q05920	1.32	0.0036	1.21	0.0004
	Very long-chain acyl-CoA synthetase	O35488	1.3	0.0368	1.3	0.0124
	Fatty acid-binding protein, liver	P12710	1.37	0.0378	1.43	0.0003

Down-regulated proteins in 5FU of both datasets in details are: i) Annexin A6; a calcium and phospholipid binding protein that plays an important role in regulating cholesterol transport, thus in hepatic lipid and glucose hemostasis (Qi et al., 2015). Moreover, it has shown a key role in mitochondrial morphogenesis and fidelity (Chlystun et al., 2013). ii) Ferritin heavy chain; an iron ion binding protein, is located mainly in the cytoplasm and has a role in storing iron as well as protecting cells from oxidative stress (Aung et al., 2007). Down-regulation of this protein in liver and up-regulation in serum is indicative of liver damage (Kell and Pretorius, 2014). iii) Protein disulfide isomerases (PDIs) A3 and A4 are ER-marker proteins that act as molecular chaperones. Suppressing the expression of PDIs led to unfolded proteins accumulating and thus ER stress, which in consequence activated the apoptotic pathways (Grek and Townsend, 2014).

Of the up-regulated proteins in both datasets, 3-hydroxyacyl-CoA dehydrogenase type-2 catalyses the third reaction of the mitochondrial  $\beta$ -oxidation cascade, playing a role in beta-oxidation pathway. Carbamoyl-phosphate synthase (CPS1) is an intramitochondrial enzyme; it is involved in the formation of carbamoyl phosphate, the first committed step of the urea cycle. CPS1 is considered a turnover marker of mitochondrial damage (Crouser et al., 2006). CPS1 gene was found up-regulated in rectal cancer, indicating its role in poor response to chemotherapy by increasing glutamine metabolism, thus energy production (Lee et al., 2014). Glycerol-3-phosphate acyltransferase 1 is an outer mitochondrial membrane enzyme that catalyzes the conversion of saturated Acyl-CoA to lysophosphatidic acid, which in ER, changed into triacylglycerol and phospholipid. Glycerol-3-phosphate

acyltransferase 1 has a protective role against oxidative stress and up-regulation in activity has been demonstrated in liver diseases including liver steatosis and fatty liver (Hammond et al., 2005, Lindén et al., 2006). Very long-chain acyl-CoA synthetase is responsible for the transformation of long-chain fatty acid into fatty acyl-CoA and carnitine in the outer membrane of the mitochondrion. Fatty acyl-CoA then binds again to carnitine forming acylcarnitine allowing it to cross the outer mitochondrial membrane. In turn, carnitine O-palmitoyltransferase 2 (CPT2) is responsible for converting acylcarnitine within to inner mitochondrial membrane back to carnitine and fatty acyl for  $\beta$ -oxidation (Nsiah-Sefaa and McKenzie, 2016). Pyruvate carboxylase (PC) catalyses the conversion of pyruvate to oxaloacetate, the essential intermediate of metabolism, linking carbohydrate, lipid, amino acid, and nucleotide metabolism (Wexler et al., 1994, Adina-Zada et al., 2012). The PC level was associated with a low level of cellular energy to support increased glycogenesis, it was also linked to the proliferation of a various type of cancer such as breast and small lung cancer (Phannasil et al., 2015, Sellers et al., 2015).

The cellular component, function distribution, and biological process of uniquely down- and up-regulated proteins by 5FU in Set A (95 proteins) and Set B (164 proteins) were examined, and then compared to a reference protein list to determine the highly enriched categories using BinGO software as illustrated in Sections 4.3.1 and 2.2.1.13.

The cellular component analysis demonstrated that uniquely down-regulated proteins by 5FU of Set A (67 proteins) and Set B (113 proteins) were categorized into 16 cellular compartments. The analysis showed that both



datasets were significantly enriched in microsomal compartments including, mitochondrion, ER, and the ribosome. Consistently, the biological processes evaluation displayed proteins involved in metabolic processes, cellular processes, and transportation. However, proteins of Set B were uniquely down-regulated in programmed cell death processes including, cell recognition, cell-cell signaling, cell communication, cell cycle, cellular hemostasis, DNA metabolism, and response to a stimulus.

On another hand, 28 and 51 proteins were uniquely up-regulated proteins by 5FU of Set A and Set B, respectively. The cellular component categories proteins into 12 groups, where most of them belonged to microsomal compartments. However, nucleus-related proteins were only identified in Set A. whilst, ribosome, lysosome, and endosome compartments were determined exceptionally in Set B. Similarly, the analysis showed that up-regulated proteins were significantly involved in lipid, amino acid and carbohydrate metabolisms and generation of precursor metabolites and energy. However, cellular process-related proteins such as cell-cell signaling, cell proliferation, cell cycle and cell death were identified uniquely for Set B in both down- and up-regulated proteins, which pointed a significant bias towards a specific group of proteins compared to Set A.

#### **4.3.5 Functional characteristics of the proteins detected in the datasets**

A number of differentially expressed proteins were chosen for further investigation of the proteomics data based on their significant ratios or their relevance to liver microsomes abundance and function.

Major urinary proteins (MUPs) were found to have significant differences (> 6 fold in DOX-treatment) in expression between Set A-treated and control liver microsomes. Interestingly, their expressions were asymmetrical in Set B, making them interesting to be investigated further since the key objective of this chapter is to identify potential biomarkers for treated liver microsomes by major anti-cancer drugs. CYP450 isoforms were also included because of: 1) their essential role in metabolizing anti-cancer drugs, and 2) their remarkable abundance in liver microsomes.

Due to the higher degree of homology within MUPs and CYP450s, two unique peptides with MASCOT score > 28 or one unique peptide with at least two spectra (MASCOT score > 28) were required for protein identification and quantification (section 2.2.1.10)

Confirming the existence of unique peptides for each isoform was performed using Skyline software (Skyline 3.5-64 bit) and the multiple sequence alignment tools (Clustal Omega 1.2.1) (section 2.2.1.13). The different sequences of MUP isoforms and their unique peptides are shown in Figure 4.8.

Accession	Protein name	Sequences
sp P04939 MUP3_MOUSE		MKLLLPLLLLLCLELTLVCIHAEESSSMERNFNVEQISGYWFSIAEASYEREKIEEHGSM
sp P11589 MUP2_MOUSE		----MKMLLLLCIGLTLVCVHAEASSTGRNFNVEKINGEWHITILASDKREKIEDNGNF
sp P11588 MUP1_MOUSE		----MKMLLLLCIGLTLVCVHAEASSTGRNFNVEKINGEWHITILASDKREKIEDNGNF
sp P02762 MUP6_MOUSE		----MKMLLLLCIGLTLVCVHAEASSTGRNFNVEKINGEWHITILASDKREKIEDNGNF
sp P11590 MUP4_MOUSE		-----MKLLLCIGLTLVCIHAEATSQGNLNVKINGEWFISILLASDKREKIEEHGSM
sp P11591 MUP5_MOUSE		----MKMLLLLCLELTLVYVHAEASSEGQNLNVKINGKWFISILLASDKREKIEEHGTM
sp P04939 MUP3_MOUSE		RAFVENITVLENSLVFKFHLIVNEECTEMTAIGEQTAKAGIYYMNYDGFNTFSILKTDYD
sp P11589 MUP2_MOUSE		<b>RLFLEQIHVLEKS</b> LVLFKHTVRDEECSELSMVADKTEKAGEYSVTYDGFNTFTIPKTDYD
sp P11588 MUP1_MOUSE		RLFLEQIHVLENSLVLFKHTVRDEECSELSMVADKTEKAGEYSVTYDGFNTFTIPKTDYD
sp P02762 MUP6_MOUSE		RLFLEQIHVLENSLVLFKHTVRDEECSELSMVADKTEKAGEYSVTYDGFNTFTIPKTDYD
sp P11590 MUP4_MOUSE		RVFVEHIHVLNSLAFKFHTVIDEGCEIFLVADKTEKAGEYSVMYDGFNTFTILKTDYD
sp P11591 MUP5_MOUSE		RVFVEHIDVLNSLAFKFHTVIDEECTEIVLVADKTEKAGEYSVTYDGFNTFTILKTDYD
sp P04939 MUP3_MOUSE		NYIMIHLINKDKGKTFQLMELYGREPDLSDIKEKFA <b>KLCEEHGILRENIIDLTNVNRC</b> L EARE
sp P11589 MUP2_MOUSE		NFLMAHLINEKDGETFQLMGLYGREPDLSSDIKERFA <b>KLCEEHGILRENIIDLSNANRCL</b> QARE
sp P11588 MUP1_MOUSE		NFLMAHLINEKDGETFQLMGLYGREPDLSSDIKE <b>RFAQLCCKHGILRENIIDLSNANRCL</b> QARE
sp P02762 MUP6_MOUSE		NFLMAHLINEKDGETFQLMGLYGREPDLSSDIKE <b>RFAQLCCEEHGILRENIIDLSNANRCL</b> QARE
sp P11590 MUP4_MOUSE		NYIMFHLINEKDGKTFQLMELYGRKADLNSDIKEKFKVLCCEEHGIIKENIIDLTNKTNRCL KARE
sp P11591 MUP5_MOUSE		NYIMFHLINKDEENFQLMELFGREPDLSSDIKEKFAKLCEEHGIVRENIIDLSNANRCL QARE

**Figure 4-8. Multiple sequence alignment of mouse MUP isoforms showing the high degree of homology. The red peptides represent the unique peptides for each MUP isoform that were identified in Sets A and B.**

Based on the aforementioned criteria, four MUP isoforms (MUP1, 2, 3 and 6) and two (MUP1 and 6) were unambiguously identified by Set A and Set B, respectively (Table 4.9). Quantitatively for Set A, 5FU and CIS resulted significantly in up-regulation of three MUP isoforms (MUP1, 2 and 6), where DOX was up-regulated the four isoforms (MUP1, 2, 3 and 6). Neither MUP1 nor MUP6 was significantly affected by 5FU administration to mice in Set B, where MUP6 was significantly down-regulated only by PAX, oppositely TCPOBOP was up-regulated both MUP1 and MUP6 (Table 4.9).

**Table 4-9. Major urinary proteins unambiguously identified in the Sets A and B. Mean iTRAQ ratios with their standard deviations (SD) were used for significance determination (p-value). MUP considers up- or down-regulated if its mean iTRAQ ratio was higher than (1+SD) or less than (1/1+SD) respectively and the p-value < 0.05. The directions of arrow indicate whether MUP was up- or down-regulated.**

Protein name	SwissProt/ UniProt accession number	M.Wt (kDa)	pI	MASCOT score	S.C %	Total peptides assigned to the protein	Unique peptides assigned to the protein	Number of spectra for unique peptide	iTRAQ value	p-value*	SD	iTRAQ value	p-value*	SD	iTRAQ value	p-value*	SD
									Set A								
									5FU			CIS			DOX		
MUP1	P11588	20.6	4.9	784	61.7	13	2	2	2.18 ↑	0.02	0.06	2.47 ↑	2×10 <sup>-2</sup>	0.86	6.27 ↑	2×10 <sup>-4</sup>	1.75
MUP2	P11589	20.7	4.9	891	66.1	13	2	5	1.81 ↑	9×10 <sup>-3</sup>	0.34	2.04 ↑	4×10 <sup>-3</sup>	0.61	2.78 ↑	1×10 <sup>-4</sup>	0.51
MUP3	P04939	21.5	4.6	112	10.9	2	2	4	1.25	0.48	0.22	1.19	0.66	0.12	2.05 ↑	9×10 <sup>-3</sup>	0.17
MUP6	P02762	20.6	4.7	792	61.7	12	1	2	1.74 ↑	0.03	0.24	2.21 ↑	0.03	0.74	3.34 ↑	3×10 <sup>-3</sup>	1.31
									Set B								
									5FU			PAX			TCPOBOP		
MUP1	P11588	20.7	4.9	437	58.9	9	1	5	1.07	0.68	0.12	1.06	0.72	0.13	1.52 ↑	2×10 <sup>-3</sup>	0.52
MUP6	P02762	20.6	4.7	485	68.3	10	1	2	0.89	0.25	0.12	0.70 ↓	9×10 <sup>-3</sup>	0.09	1.63 ↑	5×10 <sup>-3</sup>	0.47

The table shows details of identified MUPs including, their molecular weight (M.Wt), isoelectric point (pI), the average MASCOT score and the percent of sequence coverage (S.C %). (\*) indicated permuted p-value which has been computed following the permutation test.

Based on the criteria mentioned above, a total of 26 and 34 CYP450s were confidently identified and quantified in Set A and Set B, respectively (Table 4.10). The results showed that Set B recovered a number of CYP450s than Set A with higher MASCOT scores, total peptides, and unique peptides, a further indication of the more effective extraction method using combined cryo-pulverization and sonication compared to sonication alone.

Changes in CYP450 expression in response to different drugs of Set A and Set B are shown in (Table. 4.11) and (Figure 4.9). In Set A, two CYP450s (CYP2b9 and CYP2j5) were down-regulated by 5FU and none were up-regulated. While in CIS treatment, one CYP450 (CYP2c70) was down-regulated and one (CYP8b1) was up-regulated. Lastly, in Set A, two CYP450s (CYP2c50 and CYP2e1) and one CYP450 (CYP4a12a) were down- and up-regulated, respectively in DOX-treated mice. In Set B, 5FU, PAX and TCPOBOP resulted in down-regulation of five CYP450s (CYP 2a4, 2b10, 2c29, 2c50, and 3a11), fourteen CYP (CYP2a4, 2a5, 2b9, 2b10, 2c29, 2c37, 2c39, 2c50, 2c54, 2c55, 2c70, 2d10, 2f2, and 4f14) and two CYP450s (CYP2a4 and 3a16), respectively. On the other hand, one (CYP4a10) and four (CYP2c37, CYP2c39, CYP2c55, and CYP3a11) CYP450 enzyme were up-regulated by PAX and TCOPBOP, respectively. Nevertheless, none by 5FU.

**Table 4-10. Cytochrome P450 enzymes unambiguously identified in Sets A and B. The average MASCOT score and a number of common and unique peptides (plus times of observation of unique peptides) are shown. CYP isoforms were defined by at least one unique peptide with significant MASCOT score.**

Protein name	SwissProt/UniProt accession number	M.Wt (kDa)	pI	Set A					Set B				
				MASCOT score	S.C %	Total peptides assigned to the protein	Unique peptides assigned to the protein	Number of spectra for unique peptide	MASCOT score	S.C %	Total peptides assigned to the protein	Unique peptides assigned to the protein	Number of spectra for unique peptide
CYP 1a2	P00186	58.1	9.6	763	26.1	12	9	15	763	30.4	15	10	14
CYP 2a4	P15392	56.7 7	9.6	ND	ND	ND	ND	ND	1448	50.2	34	8	38
CYP 2a5	P20852	56.7	9.7	347	17.8	8	2	2	1598	55.5	36	8	27
CYP 2a12	P56593	56.1	9.7	347	17.8	12	11	15	1104	40.7	23	18	42
CYP 2b9	P12790	55.7	8	282	9.4	5	3	3	668	24.8	13	9	21
CYP 2b10	P12791	56.7	7.9	429	13.6	6	5	8	904	32.4	18	14	43
CYP 2c29	Q64458	55.7	9.4	1298	40.4	24	10	23	2092	49.4	36	12	44
CYP 2c37	P56654	55.6	6.8	739	23.9	12	2	2	1150	39.8	22	5	12
CYP 2c38	P56655	56.2	9.5	ND	ND	ND	ND	ND	695	31.0	16	4	5
CYP 2c39	P56656	55.8	9.1	815	22.2	12	3	8	1150	43.7	21	7	35
CYP 2c40	P56657	55.7	7.9	311	13.8	7	6	8	689	25.1	14	12	28
CYP 2c50	Q91X77	55.7	9.1	977	32.4	18	2	2	1875	42.0	31	4	13
CYP 2c54	Q6XVG2	55.8	7.9	899	28.8	16	4	8	1691	45.7	32	8	35
CYP 2c55	Q9D816	65.1	6.6	ND	ND	ND	ND	ND	278	17.1	6	4	7
CYP 2c70	Q91W64	56	9.1	694	27.4	12	9	21	1049	36.4	19	16	57
CYP 2d9	P11714	56.9	5.8	597	17.7	10	6	21	676	23.4	13	7	18
CYP 2d10	P24456	57.2	6.2	890	25.4	15	7	18	1362	32.3	22	10	42
CYP 2d11	P24457	57	5.8	ND	ND	ND	ND	ND	729	23.4	14	3	5
CYP 2d26	Q8CIM7	56.9	6.2	1175	34.4	19	13	31	1543	42.0	24	18	51
CYP 2e1	Q05421	56.8	9.3	1083	32.3	20	20	41	1412	44.8	30	25	82

CYP 2f2	P33267	55.9	8.8	637	26.7	12	8	14	1086	36.0	20	13	32
CYP 2j5	O54749	57.7	9.6	277	8.8	5	2	2	411	20.4	10	4	4
CYP 3a11	Q64459	57.8	9.5	496	16.9	10	6	18	893	32.5	21	10	53
CYP 3a13	Q64464	57.5	9.4	141	7.0	3	2	3	606	24.9	13	12	26
CYP 3a16	Q64481	57.8	7.7	ND	ND	ND	ND	ND	252	15.1	5	2	3
CYP 3a25	O09158	58.1	9.5	408	16.9	9	8	16	303	18.3	5	5	5
CYP 3a41	Q9JMA7	57.9	8.5	ND	ND	ND	ND	ND	867	27.6	16	7	58
CYP 4a10	O88833	58.3	9.5	ND	ND	ND	ND	ND	263	21.4	6	5	6
CYP 4a12a	Q91WL5	58.3	9.9	256	11.0	5	3	4	ND	ND	ND	ND	ND
CYP 4f3	Q99N16	59.8	9.7	74	4.0	2	2	2	112	9.7	3	2	2
CYP 4f14	Q9EP75	59.8	6.4	181	10.1	5	5	5	325	24.2	8	7	10
CYP 8b1	O88962	57.7	9.7	193	10.8	7	5	5	67	4.6	2	2	2
CYP 20a1	Q8BKE6	52.1	6.5	ND	ND	ND	ND	ND	128	11.5	2	2	3
CYP 27a1	Q9DBG1	60.7	9.7	696	25.9	12	9	17	693	34.5	14	12	19
CYP 51a1	Q8K0C4	56.7	9.3	ND	ND	ND	ND	ND	271	14.5	6	2	2

ND indicated not detected in referring set. pI, isoelectric point; S.C%, sequence coverage percent

**Table 4-11. iTRAQ ratios of identified cytochrome P450 isoforms in Sets A and B. Mean ratios of unique peptides with their standard deviations (SD) were used for significance determination (p-value). (\*) indicated the permuted p-value for CYP which has been computed following permutation test. ND indicated; Not Detected**

Protein name	SwissProt/ UniProt accession number	Set A									Set B								
		5FU			CIS			DOX			5FU			PAX			TCPOBOP		
		iTRAQ value	SD	p-value*	iTRAQ value	SD	p-value*	iTRAQ value	SD	p-value*	iTRAQ value	SD	p-value*	iTRAQ value	SD	p-value*	iTRAQ value	SD	p-value*
CYP 1a2	P00186	1.19	0.51	0.44	0.97	0.60	0.46	1.09	0.57	0.89	1.04	0.17	0.87	0.88	0.19	4×10 <sup>-4</sup>	0.85	0.18	5×10 <sup>-4</sup>
CYP 2a4	P15392	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.82	0.14	1×10 <sup>-4</sup>	0.71	0.17	1×10 <sup>-4</sup>	0.67	0.22	1×10 <sup>-4</sup>
CYP 2a5	P20852	1.27	0.33	0.35	0.97	0.19	0.58	1.07	0.27	0.98	0.97	0.18	0.22	0.74	0.17	1×10 <sup>-4</sup>	0.83	0.21	3×10 <sup>-4</sup>
CYP 2a12	P56593	1.30	0.22	0.15	1.12	0.69	0.81	1.20	0.68	0.45	1.05	0.16	0.71	1.00	0.13	0.56	0.93	0.17	0.09
CYP 2b9	P12790	0.58	0.26	0.01	0.92	0.39	0.51	0.67	0.49	0.08	0.93	0.22	0.07	0.82	0.15	3×10 <sup>-4</sup>	0.97	0.20	0.48
CYP 2b10	P12791	0.86	0.29	0.15	1.32	0.30	0.28	0.84	0.48	0.19	0.80	0.21	1×10 <sup>-4</sup>	0.68	0.23	1×10 <sup>-4</sup>	1.01	0.25	0.97
CYP 2c29	Q64458	1.33	0.57	0.06	1.25	0.27	0.27	1.24	0.37	0.29	0.82	0.27	1×10 <sup>-4</sup>	0.66	0.26	1×10 <sup>-4</sup>	0.82	0.19	1×10 <sup>-4</sup>
CYP 2c37	P56654	0.89	0.08	0.45	0.94	0.12	0.62	0.72	0.06	0.22	1.03	0.27	0.96	0.67	0.10	1×10 <sup>-4</sup>	1.36	0.20	3×10 <sup>-4</sup>
CYP 2c38	P56655	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.24	0.19	0.08	0.99	0.11	0.67	1.07	0.11	0.61
CYP 2c39	P56656	1.20	0.29	0.54	1.20	0.42	0.56	0.95	0.25	0.54	0.94	0.25	0.06	0.84	0.23	1×10 <sup>-4</sup>	1.32	0.21	1×10 <sup>-4</sup>
CYP 2c40	P56657	1.25	0.36	0.37	0.91	0.26	0.32	1.26	0.66	0.36	1.03	0.20	0.99	0.89	0.15	2×10 <sup>-3</sup>	0.88	0.18	0.01
CYP 2c50	Q91X77	1.27	0.35	0.55	1.02	0.30	0.83	0.48	0.04	0.02	0.86	0.15	8×10 <sup>-3</sup>	0.75	0.16	1×10 <sup>-4</sup>	0.82	0.12	6×10 <sup>-3</sup>
CYP 2c54	Q6XVG2	1.19	0.73	0.57	0.84	0.51	0.15	0.91	0.64	0.39	0.93	0.16	0.03	0.85	0.15	1×10 <sup>-4</sup>	0.89	0.18	0.01
CYP 2c55	Q9D816	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.95	0.10	0.35	0.56	0.17	1×10 <sup>-4</sup>	1.29	0.32	0.01
CYP 2c70	Q91W64	1.08	0.34	0.97	0.65	0.25	1×10 <sup>-4</sup>	1.22	0.80	0.38	0.97	0.14	0.16	0.80	0.14	1×10 <sup>-4</sup>	0.89	0.17	1×10 <sup>-3</sup>
CYP 2d9	P11714	1.20	0.54	0.40	1.08	0.37	0.99	1.05	0.49	0.94	0.93	0.16	0.08	0.88	0.15	5×10 <sup>-3</sup>	1.08	0.21	0.35
CYP 2d10	P24456	1.22	0.53	0.33	0.87	0.37	0.12	0.99	0.47	0.62	1.01	0.27	0.71	0.86	0.09	2×10 <sup>-4</sup>	1.01	0.19	0.96
CYP 2d11	P24457	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.97	0.14	0.55	0.93	0.11	0.32	1.02	0.09	0.95
CYP 2d26	Q8CIM7	0.95	0.41	0.21	0.82	0.29	8×10 <sup>-3</sup>	0.76	0.42	8×10 <sup>-4</sup>	1.11	0.17	0.04	0.91	0.19	2×10 <sup>-3</sup>	0.89	0.15	3×10 <sup>-3</sup>



CYP 2e1	Q05421	0.99	0.25	0.43	0.93	0.46	0.21	0.69	0.34	1×10 <sup>-4</sup>	1.06	0.18	0.51	1.07	0.19	0.23	1.04	0.33	0.52
CYP 2f2	P33267	1.27	0.35	0.24	0.94	0.36	0.37	1.26	0.52	0.31	1.01	0.26	0.78	0.84	0.21	1×10 <sup>-4</sup>	0.95	0.27	0.24
CYP 2j5	O54749	0.57	0.21	2×10 <sup>-3</sup>	0.77	0.33	0.10	0.81	0.48	0.21	1.01	0.12	0.80	0.94	0.09	0.15	0.97	0.21	0.53
CYP 3a11	Q64459	1.07	0.30	0.93	1.02	0.21	0.68	1.03	0.27	0.85	0.64	0.21	1×10 <sup>-4</sup>	0.90	0.17	4×10 <sup>-4</sup>	1.24	0.22	1×10 <sup>-4</sup>
CYP 3a13	Q64464	1.09	0.09	0.99	1.65	0.68	0.11	1.12	0.25	0.85	1.03	0.22	0.96	0.94	0.17	0.07	1.04	0.18	0.70
CYP 3a16	Q64481	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.05	0.11	0.87	0.93	0.29	0.38	0.60	0.38	2×10 <sup>-3</sup>
CYP 3a25	O09158	1.19	0.51	0.48	1.12	0.36	0.79	1.23	0.41	0.37	1.01	0.14	0.82	0.93	0.13	0.31	0.90	0.20	0.29
CYP 3a41	Q9JMA7	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.09	0.23	0.11	0.92	0.15	4×10 <sup>-3</sup>	0.94	0.22	0.11
CYP 4a10	O88833	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.91	0.17	0.87	0.88	0.19	4×10 <sup>-4</sup>	0.85	0.18	5×10 <sup>-4</sup>
CYP 4a12a	Q91WL5	0.91	0.29	0.89	1.23	0.63	0.50	2.12	0.45	2×10 <sup>-3</sup>	ND	ND	ND	ND	ND	ND	ND	ND	ND
CYP 4f3	Q99N16	1.06	0.02	0.55	0.73	0.34	0.20	0.78	0.02	0.33	1.06	0.18	0.15	1.36	0.16	2×10 <sup>-3</sup>	0.91	0.26	0.27
CYP 4f14	Q9EP75	1.01	0.60	0.10	0.72	0.26	0.06	0.74	0.47	0.11	1.01	0.01	0.81	0.93	0.19	0.45	0.90	0.20	0.45
CYP 8b1	O88962	1.07	0.30	0.97	1.74	0.56	0.03	1.07	0.39	0.99	1.07	0.22	0.84	0.83	0.11	6×10 <sup>-3</sup>	0.83	0.17	0.02
CYP 20a1	Q8BKE6	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.04	0.39	0.78	1.01	0.18	0.88	1.01	0.14	0.97
CYP 27a1	Q9DBG1	0.97	0.27	0.34	0.98	0.36	0.49	1.24	0.92	0.32	1.14	0.11	0.90	1.15	0.11	0.30	1.15	0.22	0.34
CYP 51a1	Q8K0C4	ND	ND	ND	ND	ND	ND	ND	ND	ND	0.90	0.21	0.06	1.02	0.14	0.95	1.07	0.32	0.42



## **4.4 Discussion**

Liver microsomes are mainly membrane fragments from the ER and represent an enriched source of many of the most important enzymes responsible for the modification of xenobiotic compounds including drugs. A recent attempt by Zgoda and his colleagues to profile liver microsomes from mouse, rat, and human in a comprehensive proteomics analysis demonstrated that a high proportion of identified microsomal proteins (up to 25%) belonged to integral membrane proteins (IMPs), where other compartment-related proteins were determined including proteins from cytosolic, mitochondrial, and Golgi apparatus (Golizeh et al., 2015). However, this is the first time the effect of anti-cancer drugs as well as CYP inducer drug (TCPOBOP) on liver expression has been investigated.

### **4.4.1 Identified proteins from Set A and Set B analysis**

Comparing the number of proteins identified in Sets A and B showed that 34% more proteins were identified in the latter, and of these, 6% more hydrophobic/membrane proteins were detected. This is most likely due to the improved method of proteins extraction using a combination of cryo-pulverization and sonication as deduced in Chapter 3. However, analysing particular cellular fraction raises the challenge in studying the possible pathways using an available tool such as Kyoto Encyclopedia of Genes and Genomes (KEGG), due to the possible bias in cellular location and biological processes of identified proteins.

Non-microsomal proteins were identified in Sets A and B probably due to the challenges of removing contaminants using the only ultracentrifugation for

organelle enrichment (Zanetti and Catala, 1990, Friso and Wikström, 1999, Stan et al., 2005). ER marker proteins (calnexin precursor and protein disulfide isomerase), demonstrated that both datasets were enriched with ER-related proteins (Table 4.12). In addition, more membrane-associated ER enzymes (e.g. CYP450s) were identified in Set B than Set A, which highlights the improvement of CP+S extraction method.

**Table 4-12. ER-identified subfamilies with their isoform numbers in Sets A and B.**  
**Protein families and accession were extracted from UniProt (Consortium, 2014).**

Protein class name	location	Number of Isoforms			
		Set A		Set B	
Protein production and modification					
endoplasmic reticulum resident protein (ERP)	ER lumen	2	P57759 Q9D1Q6	2	P57759 Q9D1Q6
Endoplasmin (ENPL)	ER	1	P08113	1	P08113
Nucleoside diphosphate kinase (NDK)	ER	2	P15532 Q01768	2	P15532 Q01768
Protein disulfide-isomerase (PDI)	ER	5	P09103 P27773 P08003 Q921X9 Q922R8	5	P09103 P27773 P08003 Q921X9 Q922R8
Thioredoxin domain-containing proteins (TXND)	ER	1	Q91W90	3	Q9CQU0 Q6P6J9 Q91W90
Thioredoxin (THIO)	ER	1	P10639	1	P10639
Elongation factors (EF)	ER	5	P10126 P57776 Q9D8N0 P58252 Q8BFR5	7	P10126 O70251 P57776 Q9D8N0 P58252 Q8C0D5 Q8BFR5
Heat shock cognate 71 kDa protein (HSP7C)	ER	1	P63017	1	P63017
Leucine-rich repeat-containing protein (LRC)	ER integral to membrane	2	Q505F5 Q922Q8	2	Q505F5 Q922Q8
UDP-glucose 6-dehydrogenase (UGDH)	ER	1	O70475	1	O70475
ER to Golgi vesicle mediated-transport					
Transitional endoplasmic reticulum ATPase (TERA)	ER	1	Q01853	1	Q01853
Microsomal triglyceride transfer protein large subunit (MTP)	ER	1	O08601	1	O08601
Steroid biosynthesis					
3 beta-hydroxysteroid dehydrogenase (3BHS)	ER integral to membrane	3	P26150 Q61694 Q9EQC1	1	Q9EQC1
Lipid and cholesterol biosynthesis					
Acetyl-coenzyme A acyltransferase (THIL)	ER	1	Q8QZT1	1	Q8QZT1
glyceraldehyde-3-phosphate dehydrogenase (G3P)	ER	1	P16858	1	P16858
Alpha-2-macroglobulin receptor-associated protein (AMRP)	ER	1	P55302	1	P55302
Fatty aldehyde dehydrogenase (AL3A2)	ER, membrane bounded	1	P47740	1	P47740
calcium sequestering					
Sarcoplasmic/endoplasmic reticulum calcium ATPase (AT2A)	ER integral to membrane	2	Q8R429 O55143	1	O55143
Calnexin precursor (CALX)	ER, integral to membrane	1	P35564	1	P35564
Glucose metabolism					
GDH/6PGL endoplasmic bifunctional protein (G6PE)	ER lumen	1	Q8CFX1	1	Q8CFX1
78 kDa glucose-regulated protein (GRP87)	ER	1	P20029	1	P20029

Table 4-12. continued

Xenobiotic metabolizing enzymes					
UDP-glucuronosyltransferase (UD)	ER	6	Q63886 Q64435 Q62452 Q8BWQ1 Q8JZZ0 P17717	7	Q63886 Q64435 Q62452 Q8BWQ1 P17717 Q3UP75 Q8JZZ0
Glutathione S-transferase (GST)	ER	10	P30115 P24472 Q9DCM2 P10649 P15626 O09131 P19157 P46425 Q64471 Q91VS7	10	P30115 P24472 Q9DCM2 P10649 P15626 Q35660 O09131 P19157 Q64471 Q91VS7
NADH-cytochrome b5 reductase (NB5R3)	ER, membrane bounded	1	Q9DCN2	1	Q9DCN2
Cytochrome P450 (CYP450)	ER, membrane bounded	26	P00186 P20852 P56593 P12790 P12791 Q64458 P56654 P56655 P56656 P56657 Q91X77 Q6XVG2 Q91W64 P11714 P24456 Q8CIM7 Q05421 P33267 O54749 Q64459 Q64464 O09158 O88833 Q91WL5 Q99N16 Q9EP75 O88962 Q8BKE6 Q9DBG1	34	P00186 P15392 P20852 P56593 P12790 P12791 Q64458 P56654 P56655 P56656 P56657 Q91X77 Q6XVG2 Q9D816 Q91W64 P11714 P24456 P24457 Q8CIM7 Q05421 P33267 O54749 Q64459 Q64464 O09158 Q64481 O09158 Q9JMA7 O88833 Q99N16 Q9EP75 O88962 Q8BKE6 Q9DBG1 Q8K0C4
Glutathione peroxidase (GPX1)	ER	1	P11352	1	P11352
Carboxylesterase (EST)	ER lumen	6	Q8VCT4 Q8VCC2 Q64176 Q63880 Q8VCU1 Q6AW46	6	P23953 Q8VCT4 Q64176 Q63880 Q8VCU1 Q8VCC2
Retinal dehydrogenase (AL1A1)	ER, membrane bounded	1	P24549	1	P24549

Selected ER proteins were compared to previous proteomics studies of liver microsomes (Table 4.13). The comparison demonstrated that the datasets from the current project identified a significant proportion of microsomal proteins with respect to the total number identified. Peng *et al* used alkaline carbonate to wash microsomal fractions to remove non-microsomal proteins (Peng *et al.*, 2010). The membrane proteins were enriched in the extract because of depletion of approximately 70% of the non-microsomal proteins. Hence, among 428 proteins identified, 41% were membrane-associated, compared to 24% of 259 total proteins without sodium carbonate wash (Peng *et al.*, 2010, Peng *et al.*, 2012). However, previous work on liver microsomes conducted in our lab involved alkaline carbonate for microsomal enrichment demonstrated a large reduction in the total number of identified proteins including those of microsomal origin (data not shown).

**Table 4-13. Comparison of experimental conditions and selected proteins between current study (Sets A and B) and literature data. ND; not determined**

Previous works	(Peng et al., 2010)	(Peng et al., 2012)	(Zgoda et al., 2009)	(Sutton et al., 2010)	(Mathias et al., 2011)	(Golizeh et al., 2015)	Set A	Set B
Species	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse	Mouse
Sex	Male	Male	Male	Male and female	Male	Male	Male	Female
Pre-treatment	None	None	phenobarbital	None	None	None	5FU, DOX, CIS	5FU. PAX, TCPOPBP
Sample preparation	Differential centrifugation for fractionation, followed by sucrose gradient centrifugation and alkaline carbonate treatment		Differential centrifugation for fractionation	Differential centrifugation for fractionation	Differential centrifugation for fractionation, followed by alkaline carbonate and Triton X-114 treatment	Differential centrifugation for fractionation		
Protein digestion	In-solution digestion	In-gel digestion				In-solution digestion		
Protein separation	2D-LC	2DE/1DE-SDS	1DE-SDS, 2D-LC, 3D-LC	1DE-SDS	1D-SDS	2D-LC		
Protein identification	LTQ-MS (MS/MS)	Q-TOF-MS (MS/MS)	Ion trap-MS (MS/MS)	MALDI TOF-TOF MS (MS/MS)	LTQ-Orbitrap-MS (MS/MS)	Triple TOF-MS (MS/MS)	MALDI TOF-TOF MS (MS/MS)	
Total identified proteins	428	259	4142	302	1212	1582	1146	1743
% of Membrane proteins	41%	24%	1DE:27% 2D-LC:21% 3D-LC:18%	59%	56%	18.9%	37%	34%
Selected protein superfamily								
CYP450 family members	25	10	29	27	35	29	26	34
UDP-glucuronosyltransferase	9	6	8	4	7	8	6	7
Protein disulfide isomerase	3	2	4	5	4	5	5	5
Superoxide dismutase I	1	0	1	ND	2	2	2	2
Major Urinary protein	3	2	1	ND	2	4	4	2



As the main aim of this project is to investigate the influence of anti-cancer drugs on liver microsomes, there was a greater focus on DMEs. A previous comparative proteomics study by Patterson *et al*, (Patterson et al., 2007), used in-gel digestion approach resulted in the identification of 17 CYP450 isoforms from a mouse treated with TCPOBOP. Recent studies, which used shotgun proteomics approach (SDS-PAGE free), successfully identified 27 CYP450 enzymes with a high degree of similarity, such CYP 3A, 2C and 4F subfamilies using ionic liquid-BMIM-BF<sub>4</sub> for microsomes proteins solubilisation (Sun et al., 2012). Isotope-coded affinity tagging using ICAT reagents have been used for relative quantification of cysteine-containing peptides resulting in the identification of 11 isoforms, but poor discrimination between closely related isoforms (e.g. CYP2a4/2a5) (Jenkins et al., 2006). Another comprehensive analysis of rat, mouse and human liver S9 and microsomal fractions demonstrated 29 CYP450 isoforms identified in mouse liver microsomes (Golizeh et al., 2015). Furthermore, Mathias and his co-workers solubilised the carbonate-washed microsome fraction in detergent (Triton X-114), demonstrated so far the highest proportion of identified membranous proteins (54% of total microsomal proteome) and a number of CYP450 isoforms (35 CYP450s) (Mathias et al., 2011). By using “shotgun” proteomics approaches, incorporating OG for first dimension separation in datasets A and B, we identified 26 and 34 CYP450 enzymes, respectively, which was close to that of Mathias *et al*. Furthermore, our results differentiated isoforms with very strong homology, for example, CYP2a4/5/12 in Set B compared to CYP2a5 in Mathias’ results and CYP2c38/39 in Set B compared to CYP2c39 Mathias’ results. In addition Set B contained 7 isoforms of UDP-

glucuronosyltransferase compared to 8 from Mathias' results (Mathias et al., 2011). To sum up, these results provided a large amount of high quality of data identifying microsomal proteins by Set A and Set B for studying the influence of anti-cancer drugs on mouse liver microsomes.

#### **4.4.2 Analysis of 5FU as inter-experimental control**

Analysis of the two datasets A and B identified 925 common proteins, which represent 84.4% of Set A and 53.6% of Set B. As 5FU was used in both experiments, the shared proteins and their associated 5FU/untreated iTRAQ ratios (unchanged, up- or down-regulated) were compared to determine if protein changes were replicated.

Comparison the two datasets showed very little overlap in the protein responses, with only four proteins down- and seven up-regulated in both. Whilst it was not considered significant during experimental design, this lack of correlation may have been due to the use of male mice in experiment A and female mice in experiment B. Hence, some proteins and their response to drug or toxicity may be specific to one sex. Thus, applying a statistical comparison using for example, principle component analysis (PCA) between the two datasets was not relatively useful in this situation. Previous studies have demonstrated that the expression of more than 1000 hepatic genes is sex-dependent, including DMEs, e.g., CYP450s, sulfotransferases, glutathione transferases, and UDP-glucuronosyltransferase (Waxman and Holloway, 2009, Conforto and Waxman, 2012, Buckley and Klaassen, 2007). An example of sex variation in Phase I metabolism is CYP450, a comprehensive study investigated the mRNA level of 78 CYP in different mouse tissues

including liver in male and female mice, showed that 24 and 5 CYP isoforms were predominantly expressed in female and male mice, respectively (Renaud et al., 2011). These results were consistent with our findings, where five CYP450s (CYP2a4, 2c38, 2c55, 3a16, 3a41) were exclusively identified in the female mouse experiment (Set B), whereas CYP4a12a was identified uniquely in the male mouse experiment (Set A). The Phase II DME, glutathione S-transferases (GSTP1/2) also exhibited sex variation, where GSTP1 and 2 were male predominant enzymes, but were weakly expressed in female liver (Knight et al., 2007). Consistently, our research identified both isoforms in Set A, where GSTP1 was only identified in Set B. Another example, are the solute carrier transporters (SLC), SLC22 and SLC35, whose expressions are higher in female compared to male mice (VanWert et al., 2007). Three and five isoforms of SLC were identified in Set A and Set B, respectively, among them, SLC22 and 35 were uniquely identified in the latter.

MUPs are principally synthesized in the liver and, due to their small molecular weight (19-21kDa), escape the glomerular filtration step leading to excretion in urine. Moreover, MUPs account for approximately 99% of the protein found in male mouse urine (Flower, 1996). MUPs are expressed predominantly in male mice (typically for laboratory mice 10-20 mg/ml of protein per day) compared to female mice which excreting much less (approximately 2-10 mg/ml protein per day) (Cheetham et al., 2009). Furthermore, some MUP isoforms are male-specific isoforms, e.g., MUP11 and darcin (Phelan et al., 2014). The expression pattern of MUPs in both datasets revealed more MUP isoforms identified in Set A (MUP1, 2, 3, and 6) compared to Set B (MUP1 and 6), which correlated with expected sex variation. MUP expression was much

higher in Set A compared to Set B based on the number of peptides and Mascot scores. This variation needs further investigation to exclude inter-experimental variables such as sex and control used in each Set. Therefore, the expression of MUP in treated and untreated male and female mice, for sex variation, will be investigated thoroughly in Chapter 5.

#### **4.4.3 Assessment the influence of 5FU, CIS, DOX, PAX, and TCPOBOP on liver microsome expression**

##### **4.4.3.1 5-flourouracil**

5FU has shown essential effect on RNA metabolism that contributes significantly to its toxicity (Fang et al., 2004). Previous studies showed that the levels of 28 ribosomal RNA (rRNA) were reduced to 0.19-fold by 25µm 5FU (Burger et al., 2010). The study proposed that 5FU incorporates into RNA to result in inhibition of rRNA processing. Consistently, 16 translational machinery proteins were down-regulated in our findings. Sun and his co-workers, suggested down-regulation of ribosomal genes prevents Mdm2 inactivation and p53 stabilization in 5-FU-treated cells (Sun et al., 2007)

Endoplasmic reticulum stress: Several proteins involved in protein processing in ER were down-regulated in response to 5FU, including protein disulphide isomerase (PDIs) 1a, 3a, and 4a, calreticulin and calnexin. For example, PDIs are ER stress proteins that play a crucial role in cell survival under stress condition (Zhou et al., 2008). Suppression of these proteins suggested that 5FU-induced ER stress by accumulating the unfolded proteins inside ER lumen (Yadunandam et al., 2012).

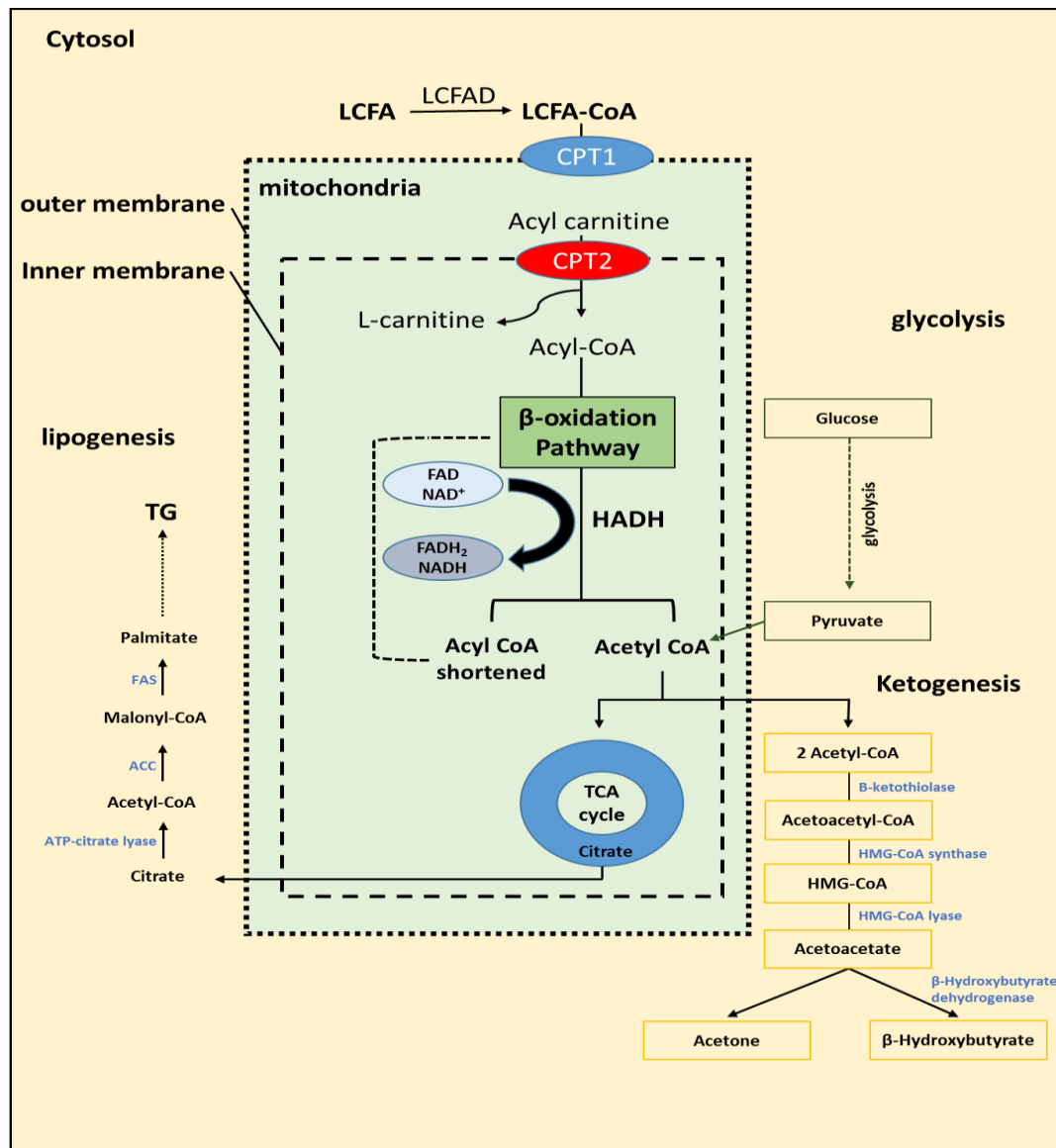
Drug metabolizing enzymes: 5FU is metabolised in the liver by CYP450s for Phase I and glutathione S-transferase (GST) for Phase II. In total, six CYP450s were down-regulated (CYP2a4, 2b9, 2b10, 2c29, 2j5, and 3a11) and none were up-regulated in responses to 5FU. Several studies demonstrated suppression of hepatic CYP2c and 2j subfamilies in rat and mice, potentiated fatty liver disease-associated hepatic inflammation and injury (Schuck et al., 2014, Anwar-mohamed et al., 2010, Li-Masters and Morgan, 2001). Therefore, modulation of CYP2c29 and CYP2j5 may be considered indicators of liver injury. Most 5FU studies have investigated CYP450 mRNA levels or enzyme activity (Giunta, 2006). A previous attempt showed that 5FU has been implicated in down-regulating the synthesis of CYP2C9 and to a lesser extent CYP3A4 (Daly and King, 2003). Another study by Afsar *et al* (Afsar et al., 1996), exhibited significant suppression in CYP2C11 and 3A content and catalytic activity in rat liver microsomes treated with one high dose (120 mg/kg, IP) of 5FU, which is very close to our experiment (100 mg/kg). Although the expression of CYP3A4 fluctuated within the duration of the experiment (14 days), the authors concluded that 5FU suppressed the expression and activity of CYP2C11 and CYP3A (Afsar et al., 1996). Our results indicate that mouse CYP3a11, which is believed to be homologous to human CYP3A4, was down-regulated. In contrast with previous observations, an alternative study used rat liver microsomes, displayed that single high dose of 5FU (120 mg/kg) did not show any changes in CYP450 expression (Stupans et al., 1995). However, they have noticed significant down-regulation in CYP2C11 and CYP3A isoforms with a consecutive low dose of 5FU (24 mg/kg/day for 5 days), suggesting that 5FU might inhibit their expressions (Stupans et al., 1995). Park

*et al*/ have used human liver microsomes to evaluate the alteration in CYP450s demonstrated no inhibitory effect of 5FU on these CYP isoforms, although there was a slight inhibition in CYP2C9 activity (Park and Kim, 2003). Accordingly, only CYP1A2 isoform was common with our finding. Recently, new cases have been reported on the interaction between capecitabine and phenytoin with 5FU confirming the inhibitory effect of 5FU on CYP isoforms (particularly CYP2C9) (Tanaka et al., 2014, Taguchi et al., 2015).

Oxidative stress and mitochondrial dysfunction: A previous study investigated the effect of 5FU on A549 cells demonstrated that it produced a significant amount of ROS. Moreover, the author showed that mitochondrial dysfunction is a pathway of 5FU to induce apoptosis (Su et al., 2014). The mitochondrial antioxidant, superoxide dismutase 1 (CuSOD), alongside with microsomal glutathione transferase 1 (mGST1) and thioredoxin domain-containing protein 5 and 15 (TXNDC5 and 15) were down-regulated by 5FU in our results, which may correlate with an increased redox imbalance. For instance, depletion of mGST1 is a hallmark to endogenous and chemically-induced oxidative stress (Lee et al., 2008, Ott et al., 2007). Further, a recent study evaluated systemically the toxic effects of 5FU and DOX on the activity of SOD1 reported down-regulation in SOD1 (Aikemu et al., 2016).

TCA cycle and  $\beta$ -oxidation pathway: several hepatic enzymes involved in tricarboxylic acid cycle (TCA cycle) and mitochondrial  $\beta$ -oxidation pathway were induced in response to 5FU, including: citrate synthase, malate dehydrogenase, and pyruvate carboxylase for TCA and (3-hydroxyacyl-CoA dehydrogenase type-2, long chain acyl-CoA dehydrogenase, trifunctional enzyme subunit alpha and beta and fatty acid synthase (FAS) for  $\beta$ -oxidation

pathway (Figure 4.10). These up-regulations suggested an accumulation of free fatty acids (FFAs) in hepatocyte (Smith et al., 2003). In addition, the fatty acid transporter, FABP which has been induced in the previous study in response to 5FU was up-regulated in our findings, suggested an instance increase cytoplasmic FFAs (Coe and Bernlohr, 1998).



**Figure 4-10. Schematic representation of the mitochondrial pathways in fatty acid, glucose, and ketone body's metabolism.** The rate of Acetyl-CoA formation is controlled by fatty acid degradation through  $\beta$ -oxidation, TCA cycle, glycolysis, and ketogenesis. The presence of LCFA in high amount in the cytosol stimulates their degradation in mitochondria, where LCFAD is responsible for activating LCFA into LCFA-CoA that only form able to enter the mitochondria. Transporters including CPT 1, 2 were involved in the transport of Acyl-CoA onto an inner membrane for  $\beta$ -oxidation. Produced Acetyl-CoA can go further for ketone body synthesis or enter TCA cycle for energy generation. An excess amount of Acetyl-CoA leads citrate, a component of TCA cycle, to export to the cytosol for lipid synthesis and storage. LCFA; long chain fatty acid, LCFAD; long chain fatty acid dehydrogenase, CPT1 and 2; carnitine acyltransferase I and II, HADH; Hydroxyacyl-coenzyme A dehydrogenase, TCA; tricarboxylic acid, HMG-CoA; 3-hydroxy-3-methyl-glutaryl-coenzyme A, ACC; Acetyl-CoA carboxylase, FAS; fatty acid synthase.



#### 4.4.3.2 Cisplatin

A previous proteomics study for evaluating the liver toxicity of CIS was performed by Cho and his workers (Cho et al., 2012a). A single dose (IC<sub>20</sub>, 282.37  $\mu$ M for 24 hours) was used to evaluate the hepatotoxicity of CIS on primary hepatocytes from rat liver. This study was similar to the current study of this project in point of dose of CIS (at low concentration), duration of treatment, organ (with respect to different models) and the analytical approach (i.e. shotgun proteomics). However, Cho *et al*, also performed transcriptional genomics analysis of regulated genes as a complement approach for proteomics.

Oxidative stress and mitochondrial dysfunction: Edwards *et al*, proved that CIS has been accumulated in mitochondria, which indicates that mitochondria as an organelle is the main target for CIS (Sharma and Edwards, 1983, Tacka et al., 2004). Study done by Garrido and his workers, revealed that CIS binds mtDNA, thus inhibition mtDNA and mtRNA synthesis and consequently caused mitochondrial damage by induction ROS (Garrido et al., 2008)

The level of antioxidants, catalase (CAT) and thioredoxin reductase 1 (TRXR1), were suppressed, which are consistent with previous studies (Bentli et al., 2013, Iraz et al., 2006). Earlier studies demonstrated that inhibiting TRXR1 by CIS is a mechanistic process, which leads to increase ROS, thus resulting in DNA damage and subsequently cell death (Saitoh et al., 1998, Sun and Rigas, 2008). Heat shock protein 70 (HSP70) which has antioxidant activity was up-regulated in CIS. A recent study demonstrated an elevation in HSPs including HSP70 have associated with formation fatty liver in mice

(Wheeler and Gekakis, 2014). Altogether, results are indicative of induction in ROS generation that proposed mitochondrial dysfunction.

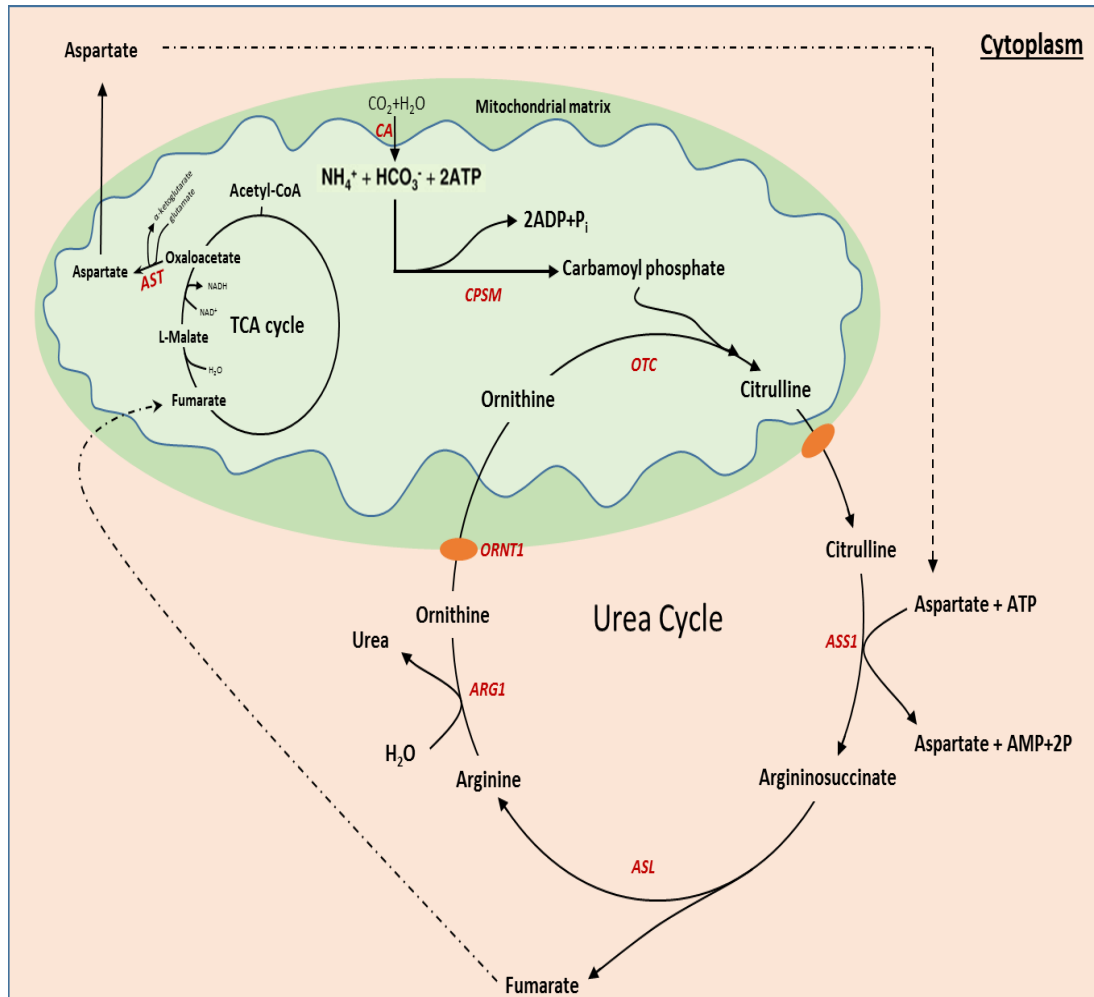
Drug metabolizing enzymes: one CYP isoform was significantly down-regulated (CYP2c70) and one was up-regulated (CYP8b1) in this project. In rat hepatocytes, Cho *et al* identified three up-regulated proteins (CYP2D1, CYP2C13, and CYP2D5) but none were down-regulated in response to CIS (Cho et al., 2012a). Hence, the role of differential expression of CYP isoforms may explain toxic metabolism of cisplatin in hepatocytes.

$\beta$ -oxidation pathway and TCA cycle FABP1 which has been up-regulated commonly in Set A was consistently up-regulated in a previous study (Cho et al., 2012a). Remarkably, our results showed up-regulation in AACS and enzymes linked to fatty acid  $\beta$ -oxidation and TCA pathways such as dihydrolipoyl dehydrogenase, DID and dihydrolipoamide S-succinyltransferase, DLST.

DID and DLST are subunits of 2-oxoglutarate dehydrogenase complex (KGDH), which is a component of TCA cycle. The decrease in KGDH level has been linked to excess ROS generated within mitochondria, due to its antioxidant property (McLain et al., 2011). In agreement with TCA suppression, the glucose transporter (Solute Carrier Family 2 Member 2, SLC2a2) was down-regulated. SLC2a2, known by Glut2, facilitates hepatocellular glucose uptake thereby regulating the expression of following enzymes involved in glucose homeostasis in liver (Meugnier et al., 2007).

Urea cycle: Three of urea cycle enzymes (Figure 4.11) were significantly down-regulated (arginase isoform 1, ARG1, argininosuccinate, ASS1,

carbamoyl-phosphate synthase, CPSM). Reduction in ASS1 has been observed previously in HepG2 cell line treated with CIS, suggesting its role in cellular sensitivity to CIS (McAlpine et al., 2014b).



**Figure 4-11. Schematic representation of the relationship Urea cycle and TCA cycle.**

Urea formation from ammonia starts inside mitochondrial matrix, which includes three steps. However, other three steps are taken place in the cytosol. The fumarate is a product of argininosuccinate lyase reaction can also be an intermediate of TCA cycle. CA3; carbonic anhydrase, CPSM; carbamoyl phosphate synthase 1, OTC; ornithine transcarbamoylase, ASS1; argininosuccinate synthase1, ASL; argininosuccinate lyase, ARG1; arginase 1, ORNT1; mitochondrial ornithine carrier isoform 1, AST; aspartate transaminase. Adapted from (Ersoy Tunalı et al., 2014).

#### 4.4.3.3 Doxorubicin

DOX accumulates mainly in the nucleus, however, notable traces of DOX were found to accumulate in the mitochondria. Alongside with the known mechanism of DOX in interfering apoptosis and survival pathways during its cytotoxic action, DOX has shown an intensive modulation in mitochondrial functions by inducing mtDNA mutations that lead to leakage of cytochrome c and the opening of mitochondrial permeability transition pores (Green and Leeuwenburgh, 2002)

Endoplasmic reticulum stress: Chaperones, PD1a1 and Calreticulin were significantly down-regulated, which indicated accumulation of unfolded proteins and thus ER dysfunction as demonstrated earlier in 5FU. In agreement with Hammer *et al* (Hammer et al., 2010), cytoskeleton proteins, cytokeratin 8 and 18 (K8/K18), profilin-1, decorin and myosin were up-regulated. These proteins have role in hepatocyte integrity in response to mechanical stress (Loranger et al., 1997)

Drug metabolizing enzymes: In this study, two CYP450s (CYP2c50 and CYP2e1) were down-regulated, where CYP4a12a was up-regulated by DOX. Zordoky and his colleagues have reported significantly two down-regulated proteins (CYP2B1 and CYP2C11) and two up-regulated (CYP1B1 and CYP4A) in rat livers treated with 15mg/kg of DOX (Zordoky et al., 2011). Overexpression in CYP4A was consistence with our results (up-regulation of CYP4A21a); however, CYP2B1 and 2B2 did not identify in the current project. Another study has been done using human liver microsomes displayed inhibitory effect for DOX on CYP3A4 (Baumhäkel et al., 2001). Di Re *et al*, investigated the capability of a pharmacological therapeutic dose of DOX

(10 $\mu$ M) to modulate CYP450s in rat liver microsomes, the results did not show any alteration (Re et al., 1999). CYP2E1, which involves the formation of ROS was significantly down-expressed by DOX, indicate a cytoprotective role of liver. Zordoky *et al* demonstrated a slight decrease in CYP2E1 expression level but not significant (Zordoky and El-Kadi, 2008).

$\beta$ -oxidation pathway and TCA cycle: Mitra *et al* have revealed that rats fed with high-fat dietary lead to sensitise cardiotoxicity induced by DOX (Mitra et al., 2008). It has been suggested that lipid peroxidation in consequence to a high level of ROS have considered as a possible mechanism of DOX induced-steatosis (Šimůnek et al., 2009, Kim et al., 0000, Fu et al., 2010, Rashid et al., 2013). A change in the expression of fatty acid metabolism was noticed in the present analysis. For instance, down-regulation of apolipoprotein E, prolow-density lipoprotein receptor-related protein 1, which have shown role in protecting liver formation steatosis (Ding et al., 2016).

Alongside with commonly up-regulated proteins with 5FU and CIS, acyl-CoA dehydrogenase and non-specific lipid transfer protein were uniquely over-expressed by DOX. These proteins that are involved in  $\beta$ -oxidation were found up-regulated previously in response to DOX (Hammer et al., 2010). (Figure 4.10).

Urea cycle: It has been known that anti-cancer drugs such DOX can affect urea cycle enzymes (El-Sayyad et al., 2009). In the present study, ASS and mitochondrial ornithine transporter 1, ORNT1, were down-regulated, where ARG1, CPS1, and carbonic anhydrase 3, CA3, were up-regulated (Figure 4.11). This disagreement in proteins alterations can be correlated to the

deleterious effect of DOX or the role of each protein in response to induced stress. For example, ARG1 was over-expressed in inflammation and high level of ROS, it has a role in activating macrophages preventing excessive nitric oxide (NO) production (Ricardo et al., 2008).

#### **4.4.3.4 Paclitaxel**

PAX is known to induce different cytotoxicity including, GI disorders, cardiac and skeletal muscle toxicity, myelosuppression, neurotoxicity, and acute liver injury (mostly hepatic cytolysis) (Foufelle and Fromenty, 2016). A recent case report showed that PAX can induce hepatic necrosis after acute dose (Mandaliya et al., 2015).

Oxidative stress and mitochondrial dysfunction: The exact mechanism by which PAX induced liver toxicity is not clear, it has been reported that PAX produces cytotoxicity by generating ROS (Tanimukai et al., 2013, Ryu et al., 2006, Xiong et al., 2014). Mitochondrial antioxidant proteins were down-regulated by PAX including, TXNDC5 and 15, catalase, GPX1. Moreover, two isoforms of GSH (omega and P1) were down-regulated. Similar to 5FU, down-regulation of these proteins indicated exaggeration in the oxidative stress. Previous studies showed that the increase in the cellular content of H<sub>2</sub>O<sub>2</sub> is the target of PAX in inducing toxic effects on cancer cells (Hadzic et al., 2010, Alexandre et al., 2007).

Numerous studies investigated the expression and activity of different antioxidant enzymes such as, SOD, catalase, GSH, and GPX in response to PAX (Pieniążek et al., 2013, Altintas et al., 2015). In agreement with our observations, these enzymes were suppressed in response to PAX.

A recent work investigated the ability of PAX-solvent (CrEL) to induce oxidative stress in liver. Results showed that both PAX and CrEL were able to alter the expression of plasma lipid peroxidation, SOD and catalase activities, (GSH) levels, thus both of them induced oxidative stress (Campos et al., 2014).

Endoplasmic reticulum stress: In the current research, the expressions of several ER-marker proteins were reduced in response to PAX, including PDI family (PDI1a1, PD1a3, PDI4a, and PD1a6), calreticulin, calumenin, and calnexin. These proteins work as chaperone to regulate protein folding, cellular responses to stress and intracellular calcium ( $\text{Ca}^{2+}$ ) levels (Liao et al., 2008). Down-regulation of these proteins may lead to aggregation of unfolded proteins and intracellular  $\text{Ca}^{2+}$  homeostasis alteration, thus resulted eventually in ER stress and cellular apoptosis (Ermak and Davies, 2002). It has not been reported the role of ER in PAX in toxicity (Liao et al., 2008). Such these proteins may suggest this role.

$\beta$ -oxidation pathway: It has been reported previously in minor cases that PAX may induce fatty liver (Harries et al., 2004). Our findings showed slight alterations in fatty acid metabolism pathway. This is including up-regulation in very long-chain specific acyl-CoA dehydrogenase (ACADV1), acyl-coenzyme A oxidase 1 (ACOX1), and 3-ketoacyl-CoA thiolase B (ACAA1b). These enzymes have role in lipid degradation (Figure 4.10), which may have suggested a slight increase in cytoplasmic FFAs content in response to either PAX or CrEL. However, the latter is a fatty acid esters of polyethylene glycol, which metabolises in the liver to produce FFAs that increase the content of hepatic lipids.

Drug metabolizing enzymes: Surprisingly, PAX had suppressed the expression of 13 CYP450s (CYP2a4, 2a5, 2b9, 2b10, 2c29, 2c37, 2c39, 2c50, 2c54, 2c55, 2c70, 2d10, 2f2, and 4f14). These changes have never been reported before in literature. However, PAX is metabolised primarily in the liver via CYP2C8, 3A4, and 3A5, in addition, it is believed that PAX modulate the expression of these isoforms in mouse (Nallani et al., 2003). Thought, PAX solvent (CrEL) has shown the ability to modulate several hepatic enzymes such as CYP3A and CYP2C9 (Christiansen et al., 2011). It has been suggested that produced FFAs such as stearate from CrEL modulated CYP450s (Zhu et al., 2009). Furthermore, CrEL is known as organic anion transporting polypeptides (OATP) 1A2, 2B1, 1B1, and 1B3 inducer (Engel et al., 2012). In our result, OATP2B1 was up-regulated. In summary, these changes required further investigation to confirm whether the alterations were due to PAX or CrEL.

#### **4.4.3.5 TCPOBOP**

TCPOBOP is a CAR agonist that has been used commonly to induce CYP enzymes (Wei et al., 2000, Kelley et al., 1985). In addition to induction of CYP450s, TCPOBOP has shown a hepatoprotective mechanism by inducing pivotal enzymes at the gene level, for example, those involved in  $\beta$ -oxidation and peroxisome fatty acid oxidation, thus ameliorating hepatic steatosis induced by xenobiotics (Baskin-Bey et al., 2007). Several studies confirmed the effect of TCPOBOP on CYP450 at gene and protein level (Baskin-Bey et al., 2007). Edwina *et al*/displayed significant up-regulation in the expression of CYP2b and CYP3a11 using mice treated with 3mg/day of TCPOBOP for 3



days. Another study on mRNA level demonstrated up-regulation in Cyp1a1, 2b10, and 39a1 and down-regulation Cyp2c38, Cyp2u1, Cyp4v3, and Cyp17a1 by using 3mg/kg/day of TCPOBOP to treat mice for two days. Additionally, a comparative proteomics study performed by Lane and her workers (Lane et al., 2007), identified 17 CYP450s in mice treated with TCPOBOP as a single dose (3mg/kg) and sacrificed after 4 days of treatment, among these isoforms. The expression of nine CYP450s (CYP1a2, 2a4/5, 2b10, 2b20, 2c29, 2c37, 2c38, 3a11 and 39a1) were significantly up-regulated, and CYP2c40, 2e1, 3a41 and 27a1 were significantly down-regulated.

In our findings, a single dose (3mg/kg for 24 hours) of TCPOBOP was used as a CYP inducer. Of regulated CYP isoforms, the expression of CYP2a4 and CYP3a16 were significantly suppressed. Oppositely, four isoforms (CYP2c37, 2c39, 2c50 and 3a11) were up-regulated. Results to some extent are with an agreement with previous studies.

Although the present work has used the similar route of administration and dose of TCPOBOP (3mg/kg) similar to previous studies, the duration of treatment was not consistent. This may explain the lesser number of induced CYP450s than expected. Likewise, proteins for instance involved in  $\beta$ -oxidation pathway were not induced in this research as observed previously by Baskin and co-workers (Baskin-Bey et al., 2007).

## Chapter five: Investigating the influence of sex and selective anticancer drugs on the expression of signature proteins in mouse liver

## **5 Investigating the influence of sex and selective anticancer drugs on the expression of signature proteins in mouse liver**

### **5.1 Introduction**

Verification of the biomarkers identified by proteomics technologies has emerged as an important step to building a reliable pipeline in biomarkers development. The verification step acts as a bridge between the biomarkers identification step and qualification for clinical trials. Western blotting is widely technique used prior proteomics studies for qualitative and quantitative validation.

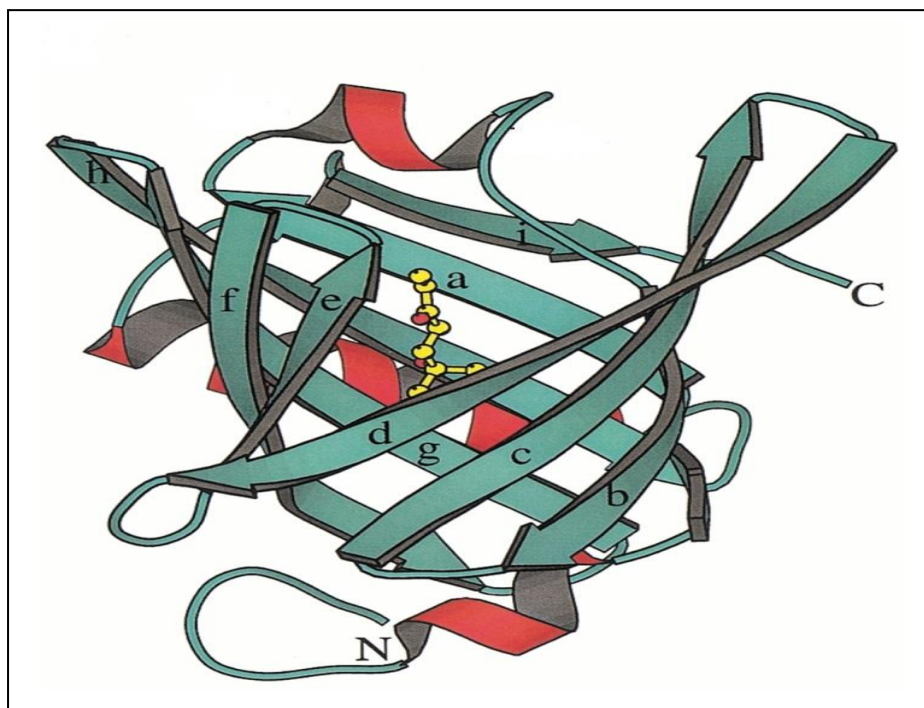
A retrospective review of the literature indicates that sex variation in drug efficiency and toxicity profiles has previously been reported (Gandhi et al., 2004, Franconi et al., 2007, Mennecozzi et al., 2015). In fact, male and female differ beyond their reproductive systems, and this difference extends to include all the physiological systems, such cardiovascular, immunological, neurological systems, metabolic function of the liver, etc. (Wizemann and Pardue, 2001). It has been reported from different studies in mouse and rat liver model that the expression of more than 1000 genes is sex dependent. A study has been undertaken using male and female mice to examine if the possible ameliorative effect of riboflavin on cisplatin-induced liver toxicity is sex dependent showed it to be more significant in the latter (Naseem et al., 2015). However, the emerging picture from the literature has indicated that less than 50% of the research studies published have reported sex as a biological variable in their experimental animals, and only 20-28% of the

studies on cell lines stated the sex of the original primary cells used (Taylor et al., 2011). The sex bias is a noteworthy problem in scientific research as it might result in serious consequences in response and toxicity of drugs on both sexes (Check, 2010).

Therefore, we explore the impact of sex variation in pharmacoproteomics in this chapter, by analysing two target protein families, Major urinary protein (MUP) and cytochrome P450 (CYP), in both, male and female mice treated with selected anticancer drugs.

Major urinary proteins (MUPs) are a subgroup of proteins, of approximately 10-15 discrete species in wild mice and 5-7 species in genetically homogeneous inbred laboratory mice, which all belong to a superfamily called lipocalins (Armstrong et al., 2005, Böcskei et al., 1992). Despite being also expressed in different glandular tissues such as salivary, nasal and mammary glands (Shahan et al., 1987b, Shahan et al., 1987a, Utsumi et al., 1999), MUPs are mainly produced in mouse liver. They are secreted by liver cells into the bloodstream to be filtered in the kidney and excreted in urine (Armstrong et al., 2005).

MUPs consist of eight-stranded  $\beta$ -sheets with additional  $\beta$ -meander connections, which together form a conserved  $\beta$ -barrel with hydrogen bonds connecting the  $\beta$ -sheets. The barrel is open at one end with a secondary structure consisting of a C-terminal  $\alpha$ -helix, which bundles outside the barrel (Figure 5.1). The  $\beta$ -barrel in MUPs is a predominantly hydrophobic cavity, which is capable of binding to lipophilic ligands (Sharrow et al., 2002).



**Figure 5-1. Structure of major urinary proteins (Timm et al., 2001). The structure illustrates the eight stranded anti-parallel beta sheets and beta meander sheet (a-i) forming the eight stranded beta-barrel structure. The carboxyl and amino termini are shown as C and N, respectively. The yellow compound represents a lipophilic ligand that binds to the hydrophobic cavity of the barrel.**

MUPs have been reported to play a key role in pheromonal communication between mice males and females. Both male and female mice deposit a range of volatile and non-volatile compounds with urine (Taylor et al., 1984), which indicate the reproductive and health status and thus, evoke sex attractiveness and communication (Zala et al., 2004, Kavaliers et al., 2005, Mossman and Drickamer, 1996). The  $\beta$ -barrel in MUPs is believed to bind to the volatile pheromones, slowing their degradation and increasing the chance for these compounds to be delivered to the olfactory system of the recipient. Also, it has been proven that MUPs can delay the release of volatile pheromones from mice scent marks, prolonging their lifetime and educing an appropriate mice behavioural response (HURST et al., 1998, Armstrong et al., 2005). MUPs

have been found to have a role not only in general attractiveness but also in mate preference by females, serving as sexually selected signals that make the female prefer a particular male over another (Kumar et al., 2014). On the other hand, MUPs can present as circulating protein in the body. These circulating MUPs have been demonstrated to have a crucial role in regulating some physiological processes, such as lipid and glucose metabolism (Zhou et al., 2009).

The expression of MUP in mouse liver is under multi-hormonal control (Knopf et al., 1983). Testosterone, growth hormone and thyroxine have been found to affect the mRNA of MUPs in the liver resulting in different isoforms (Knopf et al., 1983). For example, mice treated with testosterone have shown higher levels and more variable proportions of MUPs in urine. For instance, the authors have observed that the untreated mice that excrete MUP1 but not MUP2 appeared to excrete both isoforms after testosterone treatment, in addition to significantly induced levels of MUP1 in these mice (Szoka and Paigen, 1978). Some isoforms of MUPs are present exclusively or in significantly higher levels in males than in females (Armstrong et al., 2005). It has been reported that males' average MUP production is three to four times higher than in females (Beynon and Hurst, 2004). Furthermore, MUPs expression has been found to be influenced by health status, such as infection and immune activation (Litvinova et al., 2005, Isseroff et al., 1986), as well as the diet of the mouse (Giller et al., 2013). MUPs are encoded by different genes which are highly polymorphic and this extensive heterogeneity results in a high number of MUPs isoforms and differential ligand affinities between MUPs species (Beynon et al., 2002, Darwish Marie et al., 2001, Sharrow et

al., 2002). Accordingly, inter-individual and intra-individual variations in mice MUPs levels have been investigated and the results have shown variant and dynamic MUPs profiles, however, more proteomics studies are required to understand their significance (Thoß et al., 2015, Cheetham et al., 2009).

Cytochrome P450 (CYP450) is a group of heme-containing proteins that play a key role in phase I metabolism of drugs and xenobiotics. Sex variation in the expression of hepatic drug metabolizing enzymes, in general, is well reviewed and documented (Waxman and Holloway, 2009, Shapiro et al., 1995). In fact, CYP expression is known to be regulated by growth hormone released by the pituitary gland, which significantly shows sex variation (Shapiro et al., 1995, Cotreau et al., 2005, Kennedy, 2008). More specifically, it has been found that the expression of CYPs is influenced by the different rhythms of growth hormone secretion between males and females. For instance, growth hormone is secreted every 3.5-4 hours in males with no detectable levels between the peaks, whereas its secretion is continuous in females. This ultradian rhythm difference in growth hormone secretion between males and females has been found to be responsible for 3-5 folds difference in drug metabolism (Shapiro et al., 1995).

The superfamily P450 consists of a number of different families, which are more than 40% identical in their primary structure. The families are subdivided into subfamilies whose primary structure is more than 55% identical (Nelson et al., 1993, Gonzalez, 1988). CYP1A subfamily consists of two members in both mouse and human, CYP1A1 and CYP1A2. The two members differ in the main site and level of expression in mouse and human. CYP1A1 is an extra-hepatic enzyme that is expressed mainly in intestine, placenta, kidney and

lung (Guengerich, 1997, Baron and Voigt, 1993, Stejskalova and Pavek, 2011, Hakkola et al., 1996, Meyer et al., 2002, Cheung et al., 1999, Renaud et al., 2011). Its levels of expression are very low in human and mouse liver. In contrast, CYP1A2 is predominantly expressed in the liver with very little extra-hepatic expression levels in both mouse and human (Renaud et al., 2011). Interestingly, it has been found that CYP1A2 regulates the expression of CYP1A1 (Jiang et al., 2010, Schweikl et al., 1993). CYP1A2 is a major metabolizing enzyme in the liver, representing about 13% of liver CYP P450 content (Shimada et al., 1994).

In cancer, CYP1A enzymes have been shown to play a crucial role in susceptibility to cancer. They are responsible for detoxification and activation of polycyclic aromatic hydrocarbons (PAHs) and aromatic and heterocyclic amines, such as the compounds present in cigarettes, participating in pro-carcinogens activation (Rodriguez-Antona and Ingelman-Sundberg, 2006). CYP1A2, in particular, metabolises 4% of all drugs (Zuber et al., 2002) and consequently been shown to indirectly play a major role in breast and lung cancer. Any alteration in its expression and activity can be directly associated with increased susceptibility to cancer (Ayari et al., 2013, Seow et al., 2001). CYP1A2 activity is greatly regulated by genetic factors and, environmental factors as well as drugs and xenobiotics (Härtter et al., 2003). For example, Chloroquine (CXL), a novel anticancer drug, has been found to significantly increase the mRNA levels of CYP1A2 in hepatic cell line a dose of 10, 50 and 100  $\mu\text{mol/L}$ , which in turn decreases CXL efficacy after long-term exposure (Li et al., 2015b). Similarly, a new anticancer drug TSU-68 has shown an auto-induction of its hydroxylation (i.e. its metabolism) by increasing



CYP1A2 expression (Kitamura et al., 2008). CYP1A2 expression exhibits sex variation, with significantly higher levels of CYP1a2 protein expression in males compared to females in mice (Hersman and Bumpus, 2014). On the other hand, a female-bias was observed in the mRNA expression of CYP1A2 in human liver (Zhang et al., 2011). Based on previous observations (Chapter 4), an alteration in CYP1a2 expression has been found in response to PAX and TCPOBOP in the female set. Therefore, it was a point of interest to investigate whether this observation was due to the administration of the drugs or due to a sex variation CYP1a2 might show.

CYP2E1 is a P450 enzyme that is present in both human and mouse with 80% homologous identity in the mouse to human. CYP2E1 is expressed in different tissue such as liver, lung, and nose (Martignoni et al., 2006). In fact, it is mainly expressed in liver accounting for around 6% of the total CYP450 content in the liver (Martignoni et al., 2006, Bieche et al., 2007). Despite having an activity in some extrahepatic tissues, the activity of CYP2E1 is considered to be most fundamental in the liver as it comprises around 50% of hepatic mRNA (Bieche et al., 2007)

The high interest in studying CYP2E1 revolves around its essential role in metabolizing and activating a number of xenobiotics (e.g. ethanol) and 2% of all drugs (e.g. chlorzoxazone) (Rodriguez-Antona and Ingelman-Sundberg, 2006, Tanaka et al., 2000, Zuber et al., 2002). This enzyme is implicated in the conjugation of the drugs and xenobiotics by introducing the sites or unmasking them for subsequent elimination (Porubsky et al., 2008). Furthermore, CYP2E1 has a physiological role in starvation by metabolizing

the fatty acids and converting ketones to glucose, relating its function to diabetes and obesity (Lieber, 1997, Hong et al., 1987)

However, in the process of metabolism, toxic and carcinogenic products might be produced leading to liver toxicity and cancer. Interestingly, CYP2E1 has been found to be more prone to form ROS compared to other CYP P450 enzymes (Ioannides, 1996, Bell and Guengerich, 1997). This high propensity was suggested to be due to the unstable nature of CYP2E1 and the weak connection of the active site with other parts of the protein, which makes the substrate easy to dissociate (Porubsky et al., 2008).

The high tendency to form ROS has resulted in CYP2E1 having a critical role in determining the susceptibility of individuals to cancer and toxicity (Porubsky et al., 2008). Moreover, the special attributes of CYP2E1 are also highlighted by its high conservation among species and the absence polymorphisms which affect its expression and activity (Porubsky et al., 2008). Therefore, environmental factors and drugs that may induce the expression of CYP2E1 have grown to be of high importance, playing a major role in inter-individual variation in cancer susceptibility in reactions catalysed by this enzyme. CYP2e1 has shown significant changes in expression in the previous data (Chapter 4) in response to DOX in the male set. Such observation and the emerging picture from the literature regarding the crucial role that CYP2e1 plays in cancer drew us to shed the light on it and extensively explore the changes in its expression in response to drugs taking sex variation into consideration.

Sex variation in the expression of hepatic CYP2E1 in general, and at the protein level in particular, has not been extensively studied. Previously, hepatic CYP2e1 mRNA expression has been found to be higher in female mice than in males, and the female sex hormones have been found to induce the expression of CYP2e1 (Konstandi et al., 2013). However, when studying the expression of hepatic CYP2e1 at the protein level in mice, a previous study has shown no difference between males and females (Hersman and Bumpus, 2014).

Among CYP450, CYP3A subfamily is the most important drug metabolizing enzyme in human, accounting for 30% of CYP450 total content in human liver and responsible for metabolizing more than 50% of drugs (Martignoni et al., 2006, Zuber et al., 2002). CYP3A consists of four isoforms, 3A4, 3A5, 3A7, and 3A43 in human. CYP3A4 is the most abundant in human liver and, to a lower extent, in the intestine (Nelson et al., 2004). In previous comparison study employed six different species, mouse was proposed as the most similar to humans with respect to catalytic activities of the CYP3A subfamily (Bogaards et al., 2000). In mouse, there are six isoforms of CYP3a, CYP3a11, CYP3a13, CYP3a16, CYP3a25, CYP3a41 and CYP3a44. Where CYP3a11 is the most homologous to human sharing 76% of the amino acids of CYP3A4 (Yanagimoto et al., 1992).

The expression of CYP3A4 has been reported to display a high inter-individual variation and this variation has been mainly attributed to genetic factors (Özdemir et al., 2000). However, in the previous data (Chapter 4), five CYP3a isoforms have been identified in females, while only 3 have been identified in males. Moreover, the changes in the expression in response to drugs were

primarily observed in females. Sex variation has been shown to contribute to 5.69% variation of CYP3A4 mRNA expression in human liver (Lamba et al., 2010). While some studies have shown higher mRNA expression of CYP3A4 in human liver of females than in males (Wolbold et al., 2003, Gorski et al., 1998), other studies have not found such difference (Schmucker et al., 1990, George et al., 1995a). In regard to CYP3a11 in mouse, no significant effect has been found at mRNA level in response to continuous growth hormone treatment, suggesting a sex-independent pattern in hepatic CYP3a expression (Cheung et al., 2006). Therefore, the previous data and studies have led CYP3a to be of high interest.

A particular challenge for studying mouse CYPs using Western blotting techniques is the virtual absence of commercially available anti-mouse CYP antibodies. An exception is anti-mouse CYP2e1, but for CYP1a2 and CYP3a, anti-human equivalent CYPs will be tested for their ability to detect mouse CYPs.

### **5.1.1 Aims of the chapter**

One of the variables of the proteomics discovery work was that Set A was performed on male mice, whilst Set B was performed on female mice (Chapter 4). In the original experimental design, sex was not expected to be a significant factor affecting protein expression when testing anti-cancer drugs. Moreover, in order to state the real causes of the significant alteration in MUP expressions in Chapter 4. This project employs new Sets of male and female mice treated with DOX and PAX.

The first part of this chapter aimed to use liver microsomes from datasets, A and B, to verify the changes in MUP expression observed in the previous Chapter (Chapter 4) by western blotting technique.

The second part intended to employ new Sets of untreated and treated (DOX and PAX) liver microsomes from male and female mice, which will be used further to:

- a. Determine whether antibodies against human CYPs 1A2 and 3A4 can be used to detect equivalent mouse CYP isoforms
- b. Investigate sex variation in MUP and CYP450 (CYP1a2, 2e1, and 3a) expressions, which will help us understand some of the physiological processes in both male and female.
- c. Investigate the effect of DOX and PAX on the expression of MUP, CYP1a2, and 2e1.
- d. Evaluate the sex variation in MUP, CYP1a2, and 2e1 in response to PAX and DOX treatments.

## **5.2 Material and Method**

### **5.2.1 Animals maintenance**

Male and female BALB\cOLaHsd –Foxn1nu immunodeficient nude mice (Harlan Laboratories, UK) aged 10 weeks were used for both untreated and treated experiments. All mice were kept and maintained as mentioned in Section 3.2.1.

### **5.2.2 Cell lines**

Human colon adenocarcinoma (DLD-1) cell line was used to induce tumour in treated mice (Section 4.2.2.2).

### **5.2.3 Tumour system**

Tumours were induced only in mice intended to be treated with either DOX or PAX as described previously in Section 4.2.2.3.

### **5.2.4 Chemotherapy treatment and experimental design**

For the first part of this Chapter, prepared samples of Sets A and B in the previous chapter (section 4.2.2.4) were used. In the second part of this Chapter, new sets of sample were prepared as follow; a group of male and female mice were divided into three groups for each sex; the first group was received no treatment (NT), the second group was treated with DOX and its solvent (Normal saline - NS), and the third group was treated with PAX and its solvent (CrEL, DMSO and Arachis oil - PS) (Table 5.1).

For treated groups, mice were treated in two separate experiments; Female Mice Set (FMS) and Male Mice Set (MMS), then sacrificed 24 hours after treatment. Animals' treatment and tissues collection were done by Home Office-certified expert Tricia Cooper, at the Institute of Cancer Therapeutics, University of Bradford. Heart, lung, liver, kidney, and tumour are recovered and stored at -80°C.

**Table 5-1. Conditions of mice treatment, type of drugs and tumors used. NA indicates not applicable**

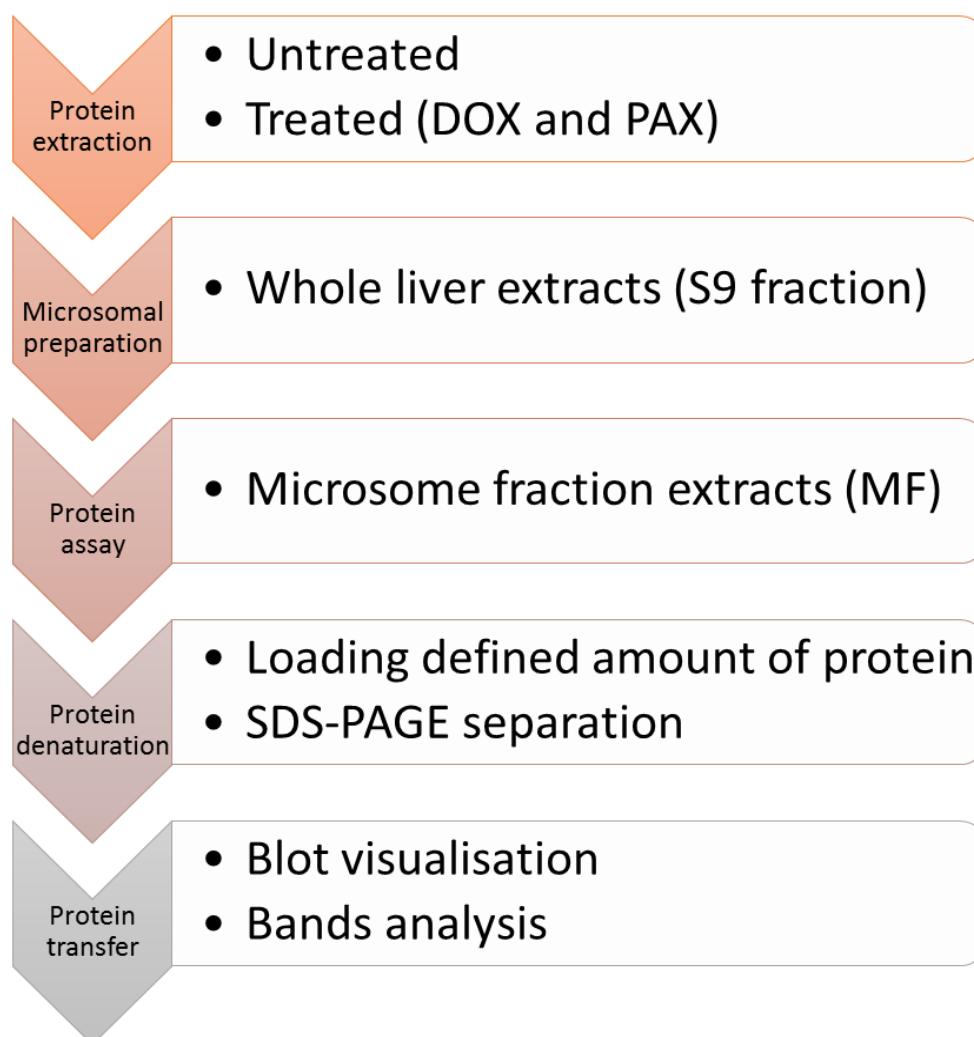
Set	Sex	Drug	Tumour type	Dose (mg/kg)	No. of mice	Route of drug administration	Duration (hr.)
<b>No treatment mice set (NT)</b>	NT-Female	NA	NA	NA	3	NA	NA
	NT-Male				3		
<b>Female mice set (FMS)</b>	FMS-DOX	DOX	DLD-1	10	3	IP	24
		NS	DLD-1	--	2	IP	24
	FMS-PAX	PAX	DLD-1	20	3	IP	24
		PS	DLD-1	--	3	IP	24
<b>Male mice set (MMS)</b>	MMS-DOX	DOX	DLD-1	10	3	IP	24
		NS	DLD-1	--	3	IP	24
	MMS-PAX	PAX	DLD-1	20	3	IP	24
		PS	DLD-1	--	3	IP	24

## 5.2.5 Sample preparation

### 5.2.5.1 Protein extraction from mouse liver by cryo-pulverization and microsomes preparation

Whole liver extracts (S9 fraction) were prepared following the optimised method; cryo-pulverization and sonication extraction (CP+S) method as described previously in Section 3.2.2.1.2. Subsequently, microsomal fractions

were prepared from the S9 fraction by means of ultracentrifugation method (section 2.2.1.1). (Figure 5.2).



**Figure 5-2. Flow diagram showing the steps of validating signature proteins by Western blot technique. After drug treatment in two independent sets (FMS and MMS), proteins were extracted by the optimized method (CP+S). Then equal amounts of proteins (40µg) were prepared for SDS-PAGE separation. Protein expression was determined via Western blot technique and band intensity analyser.**

### 5.2.6 Western blotting

Defined amount of microsomal proteins were used for Western blotting analysis as illustrated in Section (2.2.2). The conditions and concentrations of



primary and secondary antibodies that have used to validate the expression of MUP, CYP1a2, CYP2e1, CYP3a, and  $\beta$ -actin were demonstrated in Section 2.2.2.5.

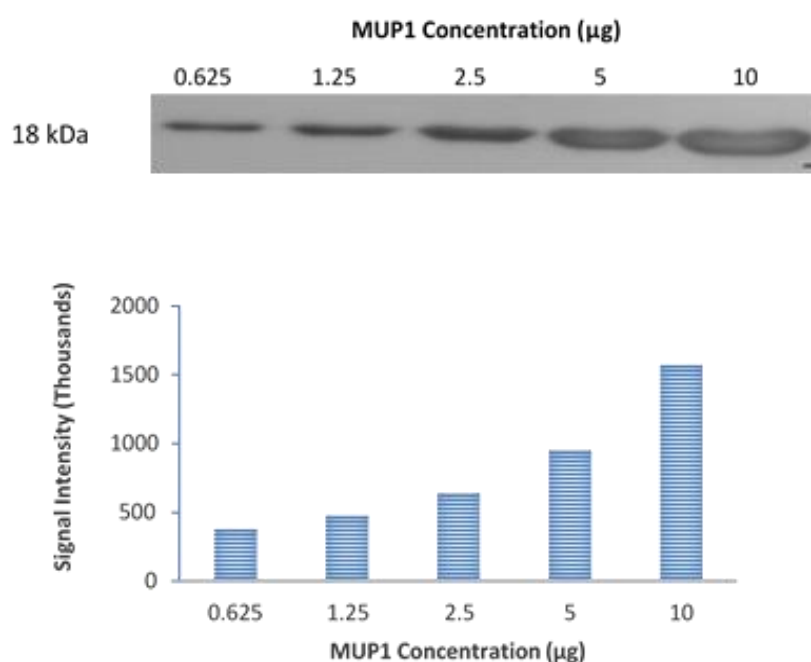
### **5.2.7 Data analysis**

Statistical differences in protein quantity between two groups were determined with an unpaired two-tailed Students' t-test (GraphPad Prism 7.0 software). For more than two group statistical comparisons, ANOVA was used. Data are presented as means  $\pm$  standard deviation (S.D.). A p-value of  $\leq 0.05$  was regarded as significantly different from control values and is shown in Figures with an asterisk.

## 5.3 Results

### 5.3.1 Optimisation of MUP1 antibody

Serial dilution of recombinant MUP1 protein (10, 5, 2.5, 1.25, 0.625 $\mu$ g) was used to evaluate the efficiency of MUP1 antibody, as well as for liver MUP quantification purpose (Figure 5.3).

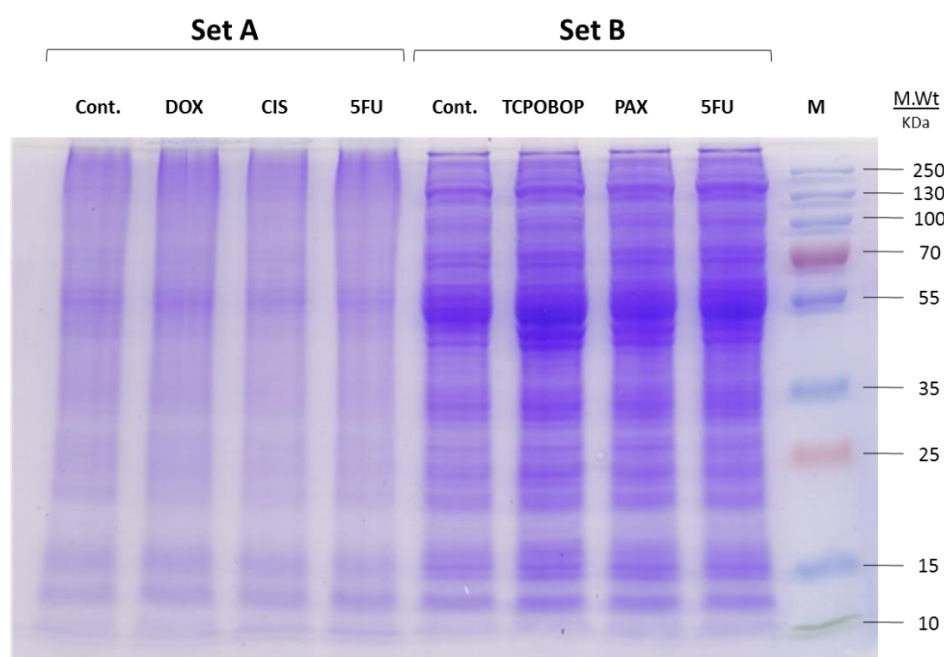


**Figure 5-3. Western blot analysis using serial dilutions of MUP1 recombinant protein.**  
Band intensity was measured via ImageJ software for each concentration and represented in barograph.

In accordance with MUP1 antibody supplier (Santa Cruz), and due to the high homology within MUP isoforms, this antibody has cross-reactivity among other isoforms of MUP. Therefore, the term MUP will represent all the possible MUP isoforms that may be detected, not just MUP1.

### 5.3.2 Validation MUP as a potential biomarker from quantitative proteomics datasets A and B

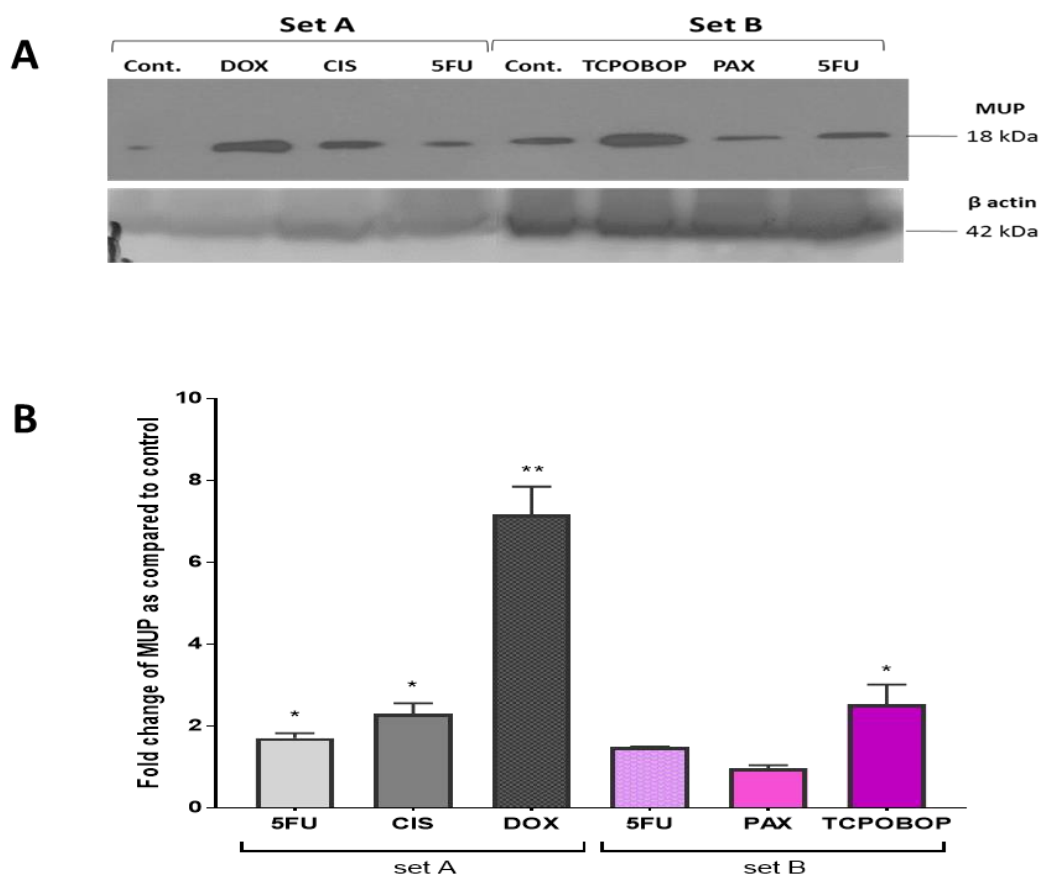
Qualitative assessment was performed first to evaluate the consistency of sample preparation and protein quantification using SDS-PAGE gel (stained with Coomassie blue) approach for liver microsomes from Sets A and B (Chapter 4) (Figure 5.4)



**Figure 5-4. SDS-PAGE analysis stained with Coomassie blue. Gel shows the efficiency of microsomal proteins preparation from sets A and B. Loading amount was 40µg proteins per lane. Lane M, Molecular weight marker**

The stained gel demonstrated good quality and reproducible lysates for Set B which has been prepared recently for this project. In contrast, Set A which has been prepared before and kept at -20°C till used at this time has shown evidence of degradation due to lack of clearly defined bands, although the amount of loaded proteins was increased to double (data not shown).

Nevertheless, a preliminary experiment was performed on Set A along with Set B to determine if these samples could be used for MUP expression by Western blot analysis (Figure 5.5).



**Figure 5-5. Effect of drugs from sets A and B on the expression of MUP in liver microsomes. Western blotting analysis in duplicate (A).  $\beta$ -actin in the same microsomal extract was used as an internal reference. Optical density reading values of the MUP protein was normalized using the expression of  $\beta$ -actin, then represented as a fold in MUP expression in compared to corresponding control (B). Number of analyses = 3. P-value was calculated following Students' t-test by comparing each treated drug with its control.**

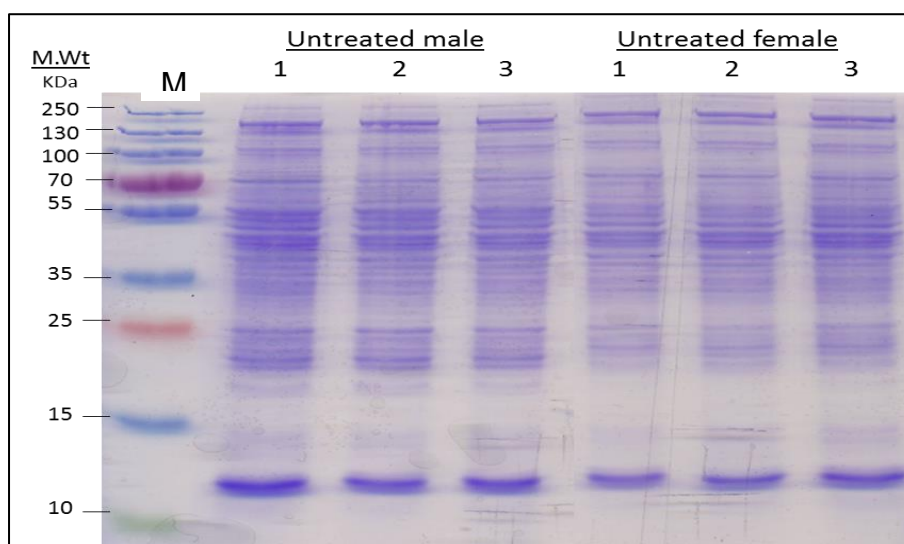
Taking into account the possible activity of MUP1-antibody against other close homology MUP isoforms, Western blot analysis showed good agreement with iTRAQ proteomics analysis (Chapter 4). Statistical analysis showed that the induction of MUP expression was significant (p-value <0.05) in Set A (5FU,

CIS, and DOX) and Set B (TCPOBOP). For Set A, male mice, liver treated with DOX showed the greatest induction in MUP expression (7.2-fold increases compared to control) followed by CIS (2.3-fold) then 5FU (1.3-fold). In Set B, female mice, TCPOBOP (2.5-fold) has significantly induced MUP expression. Moreover, consistent with proteomics data, 5FU and PAX did not induce MUP expression.

### 5.3.3 Assessing the influence of sex variation on MUP and CYP expression

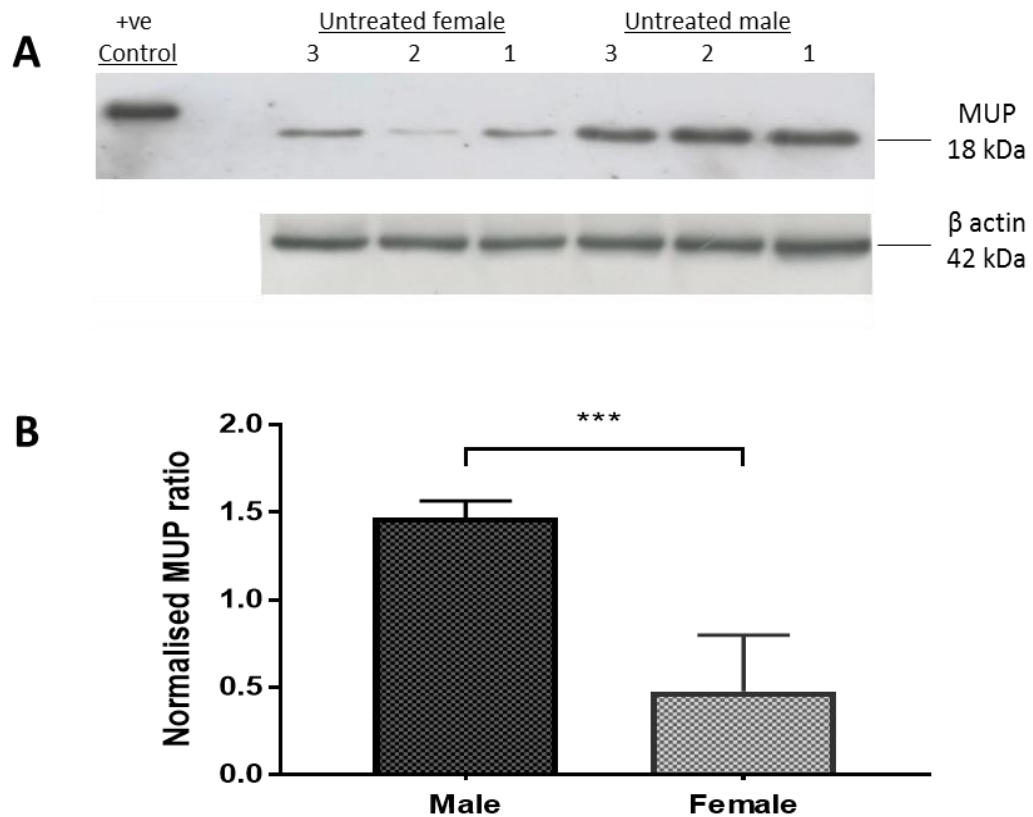
#### 5.3.3.1 Sex variation and MUP expression

A new set of untreated (NT) male and female livers were employed to investigate the differences in MUP expression. Initially, a stained gel with Coomassie blue was performed to assess the overall preparation method for untreated samples (Figure 5.6).



**Figure 5-6. Coomassie blue stained SDS-PAGE gel showing the efficiency of microsomal proteins extraction from no treatment (NT). Three mice were used for each sex. Loading volume was 20µg proteins per lane. Lane M, Molecular weight marker**

The stained gel showed good qualitative separation based on clearly defined bands, enabled used the samples for MUP expression evaluation (Figure 5.7).



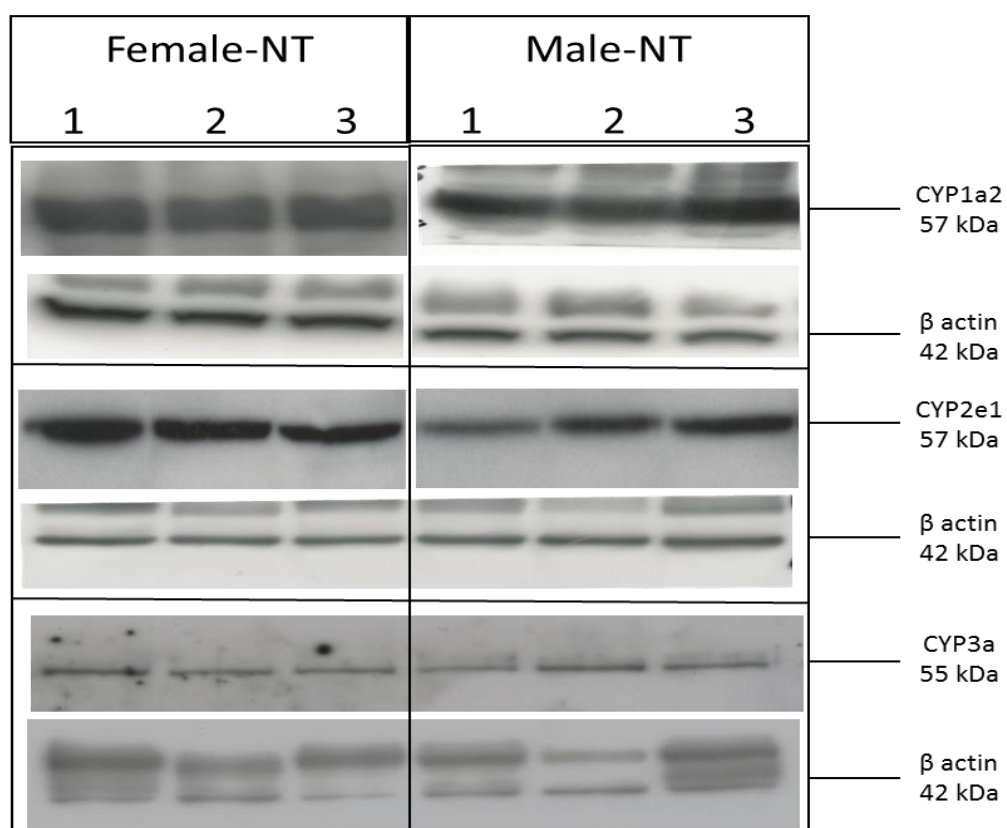
**Figure 5-7. Comparison of MUP expression between no treatment – NT, male (n=3) and female (n=3) sets in triplicate. Western blotting analysis (A).  $\beta$ -actin in the same liver extract was used as an internal reference. Optical density reading values of the MUP protein was normalised using the expression of  $\beta$ -actin, then represented as a normalised ratio of MUP expression (B). P-value was calculated following Students' t-test by comparing untreated male mice with untreated female mice. +ve control indicates MUP1 recombinant protein (1.25 $\mu$ g) included as positive standard.**

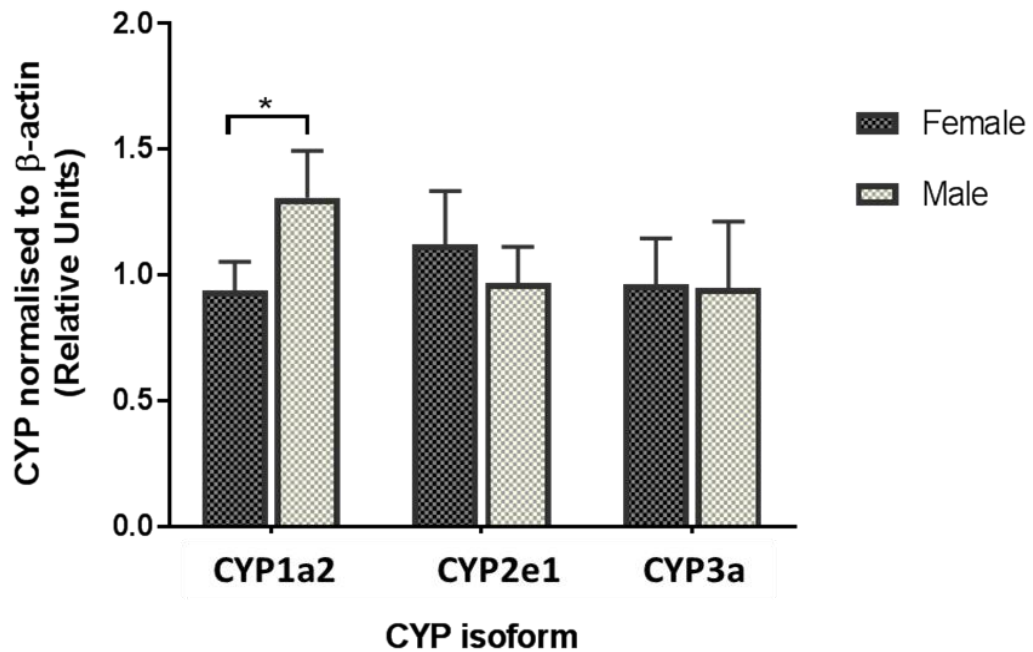
Comparing MUP expression between male and female mice received no treatment, indicated a significant increase (> 3 fold) in the male mice with greater consistency amongst the three mice (SD=0.1) compared to female mice (SD=0.32). This indicates that MUP is chiefly expressed in male mice compared to female mice.

### 5.3.3.2 Sex variation and CYP450 expression

CYP isoforms (CYP1a2, CYP2e1, and CYP3a) were selected to assess sex-based variation, which consequently will be used to investigate the capability of two anti-cancer drugs (DOX and PAX) to modulate their expressions. At the same time, this was an opportunity to determine if anti-human CYP antibodies could be used to detect mouse equivalent enzymes. The expression profiles of CYP1a2, 2e1, and 3a were evaluated in the liver of NT-female and -male mice (Figure 5.8)

**A**



**B**

**Figure 5-8. Comparison of CYP1a2, CYP2e1, and CYP3a expressions between untreated (NT) male and female. Three mice of each sex were used in Western blotting analysis in duplicate (A).  $\beta$ -actin in the same liver extract was used as an internal reference. Optical density reading values of the CYP isoform was normalized using the expression of  $\beta$ -actin, then represented as a normalised ratio of CYP expression (B). P-value was calculated following Students' t-test by comparing untreated male mice with untreated female mice for each CYP enzyme.**

Western blotting analysis exhibited a significant decrease in CYP1a2 expression in NT-female (p-value, 0.042) compared to male one. For CYP2e1 and CYP3a, the analysis did not show any significant sex dimorphism. However, CYP2e1 expression in NT-female was lower but not significant (p-value, 0.258) compared to the NT-male set.

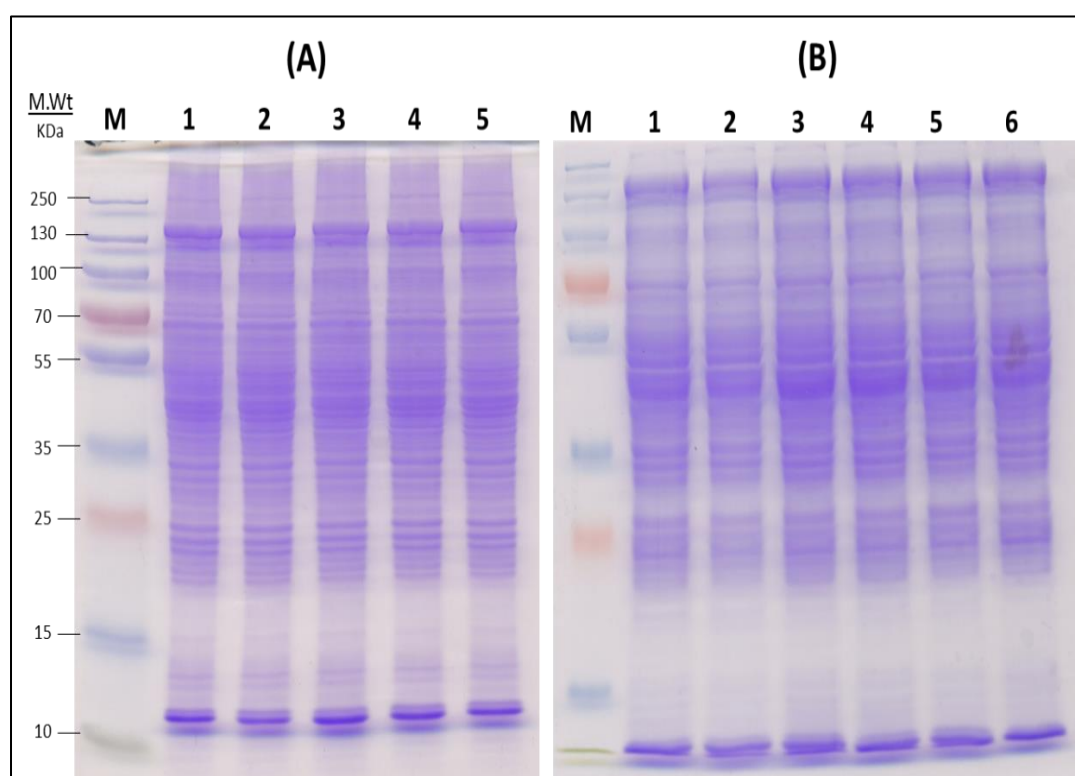


### 5.3.4 Assessing the influence of selected drugs on MUP and CYP450 expression

#### 5.3.4.1 Sex effect of treated mice with DOX on MUP expression

DOX exhibited the highest effect on MUP induction in the proteomics dataset

A. Initially, SDS-PAGE was prepared first for male and female sets to ensure the quality of microsomal proteins preparation and quantification prior Western blot analysis (Figure 5.9).

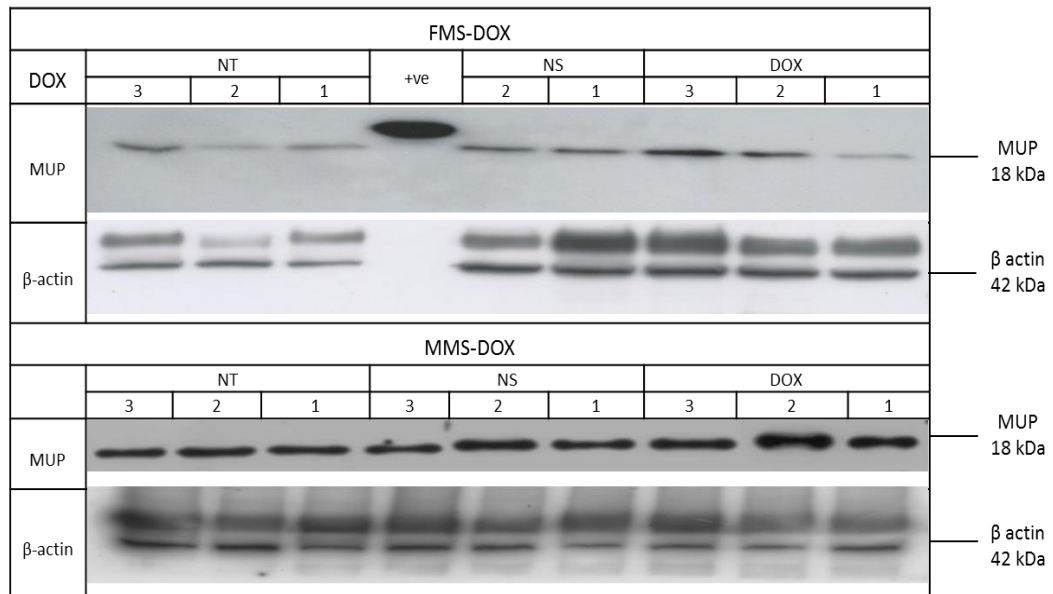


**Figure 5-9. SDS-PAGE analysis for DOX-treated female mice (FMS-DOX, gel A) and male mice (MMS-DOX, gel B). 20 $\mu$ g of protein was loaded into each lane, then separated proteins were stained using Coomassie blue stain. For both gels (A and B), Lane M, Molecular weight marker; Lane 1, DOX mouse-1; Lane 2, DOX mouse-2; Lane 3, DOX mouse-3; Lane 4, NS mouse-1; Lane 5, NS mouse-2; Lane 6, NS mouse-3 (gel B only).**

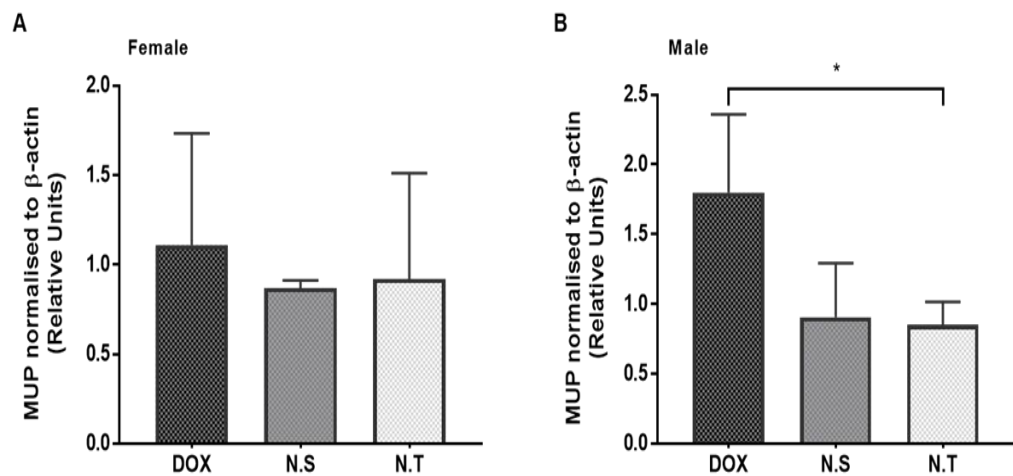
MUP expression was evaluated using Western blot analysis (Figure 5.10). In FMS-DOX and similar to the NT-female set, the amount of MUP in samples treated with DOX exhibited inconsistency in MUP expression relative to beta-actin. For samples treated with DOX-solvent (i.e. normal saline – N.S), the level of MUP was more consistent between the two mice. However, comparing the average amount of MUP for DOX-treated samples and NS-treated samples with NT samples did not show any significant variation in MUP expression. These results indicated that the expression of MUP in female mice is very low, where a slight variation in MUP expression within samples can be exaggerated statistically making it challenging to observe the effect of the drug.

In contrast, male treated mice with DOX, NS, and NT showed more consistency in MUP expression and a statistically significant up-regulation of MUP expression in DOX-treated mice (p-value, 0.0478) compared to NT, which was in agreement with the previous result in Chapter 4 (Set A). Moreover, there was no significant difference in MUP between solvent (NS) and NT, confirming that this up-regulation is in response to DOX-treatment.

I



II

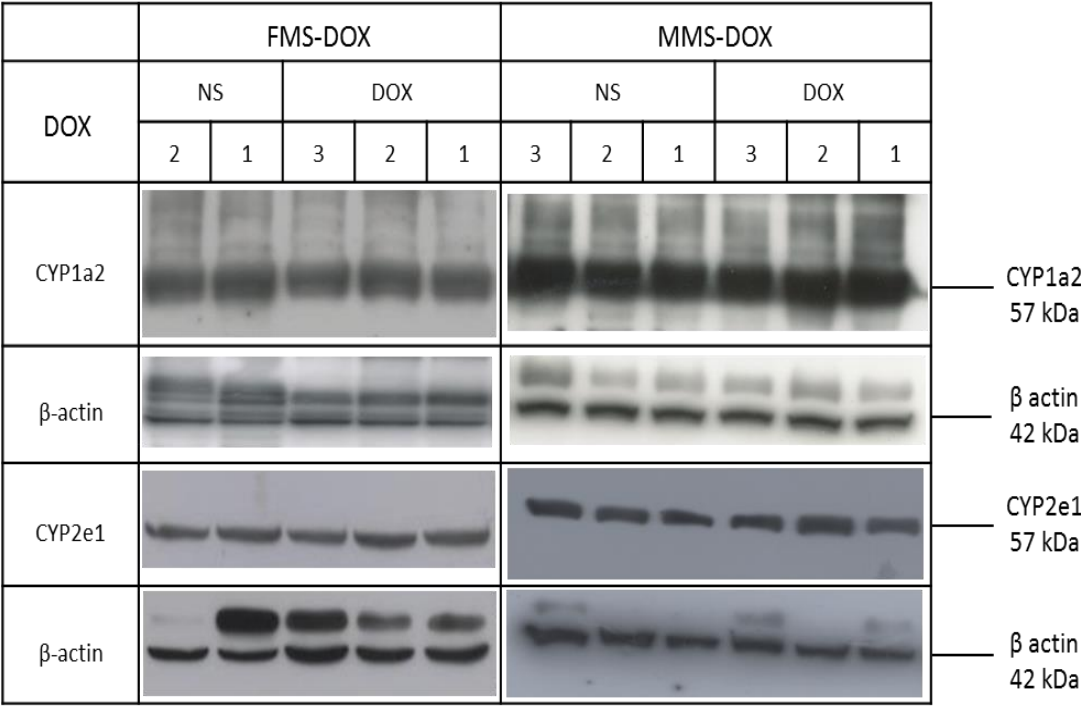


**Figure 5-10. Comparison of MUP expression between FMS-DOX and MMS-DOX.** Samples were treated with; DOX (3 mice for each set), NS (2 and 3 mice for female and male sets, respectively), and NT (3 mice for each set) in triplicate for western blotting analysis (I).  $\beta$ -actin in the same liver extract was used as an internal reference for relative expression. Optical density reading values of the MUP protein was normalized using the expression of  $\beta$ -actin, then represented as a normalised ratio of MUP expression (II). P-value was calculated following Students' t-test by comparing treated samples with their corresponding control (NT), for female Set (A) and male Set (B). +ve is indicated an MUP recombinant protein that was included as positive standard.

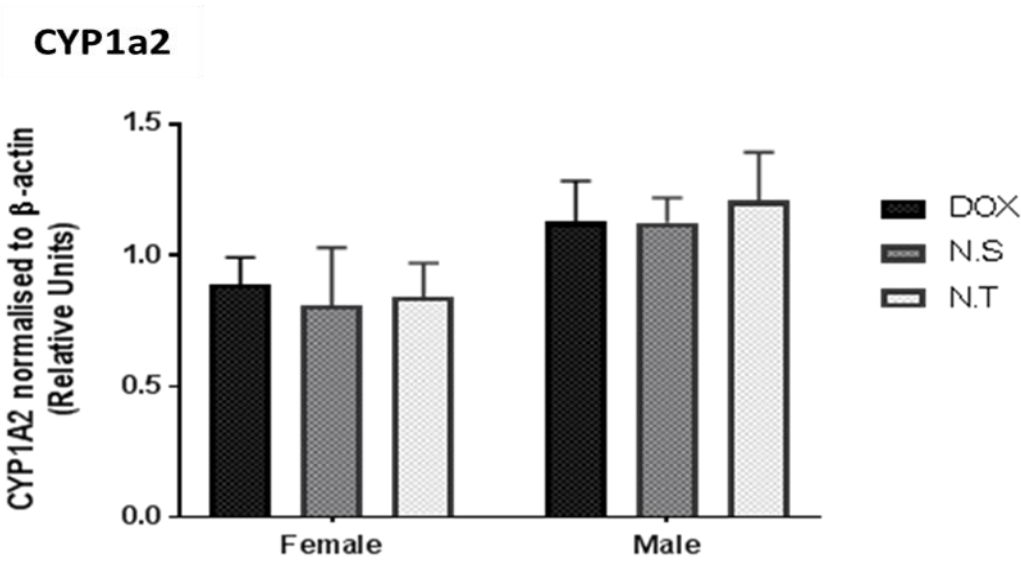
### 5.3.4.2 Sex effect of treated mice with DOX on CYP450

The effects of DOX on CYP1a2 and CYP2e1 from both sexes were investigated by Western blotting approach (Figure 5.11).

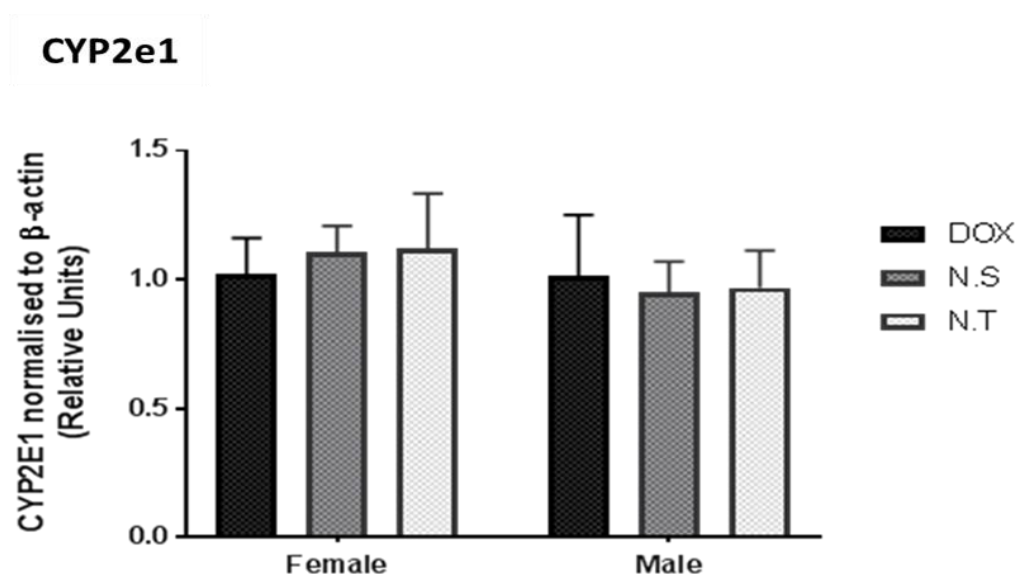
**A**



**B**



C



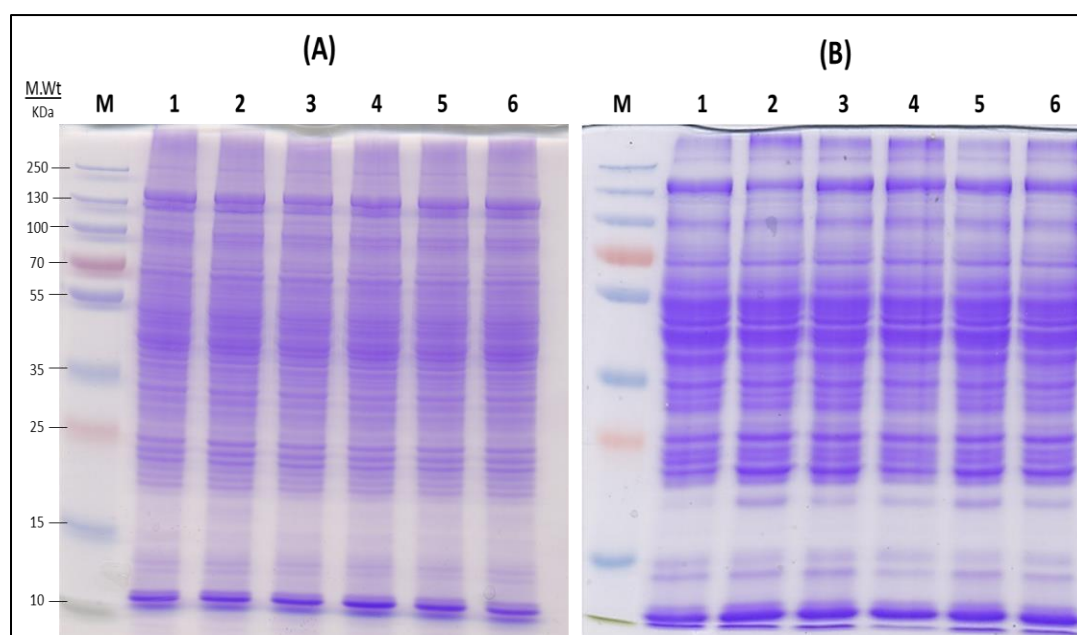
**Figure 5-11. Comparison of CYP1a2 and CYP2e1 expressions between FMS-DOX and MMS-DOX. Samples were treated with; DOX (3 mice for each set), NS (2 and 3 mice for female and male sets, respectively), and NT (3 mice for each set) in duplicate for western blotting analysis (A).  $\beta$ -actin in the same liver extract was used as an internal reference. Optical density reading values of the CYP protein was normalized using the expression of  $\beta$ -actin, then represented as a normalised ratio of CYP1a2 (B) and CYP2e1 (C) expressions. P-value was calculated following Students' t-test by comparing treated samples (DOX or NS) with their corresponding control (NT).**

The results demonstrated that DOX did not modulate either CYP1a2 or CYP2e1 expression. Moreover, the expression of both CYP isoforms was similar in DOX-treated samples from FMS and MMS.

#### 5.3.4.3 Sex effect of treated mice with PAX on MUP

The effect of PAX was only observed in the proteomics treated (Set B), where the expression of MUP1 in response to PAX was significantly down-regulated. In this analysis, the effect of PAX and its solvent (CrEL, DMSO, and Arachis oil) on MUP expression from female (FMS-PAX) and male (MMS-PAX) mice

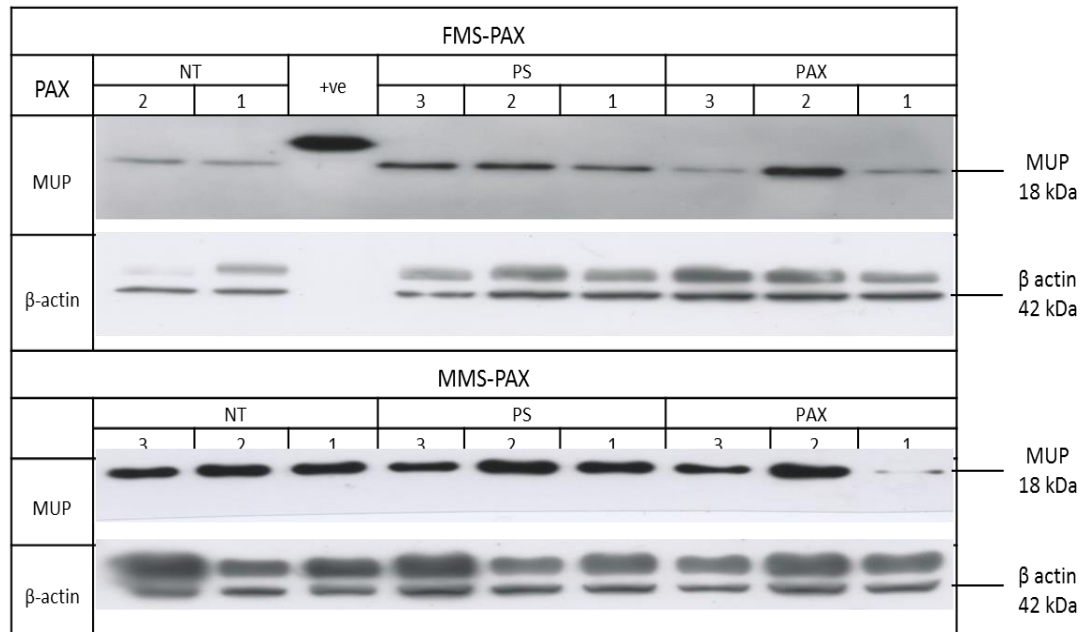
will be investigated thoroughly. Stained SDS-PAGE gels showed high quality of protein preparation and quantification in both sets (Figure 5.12)



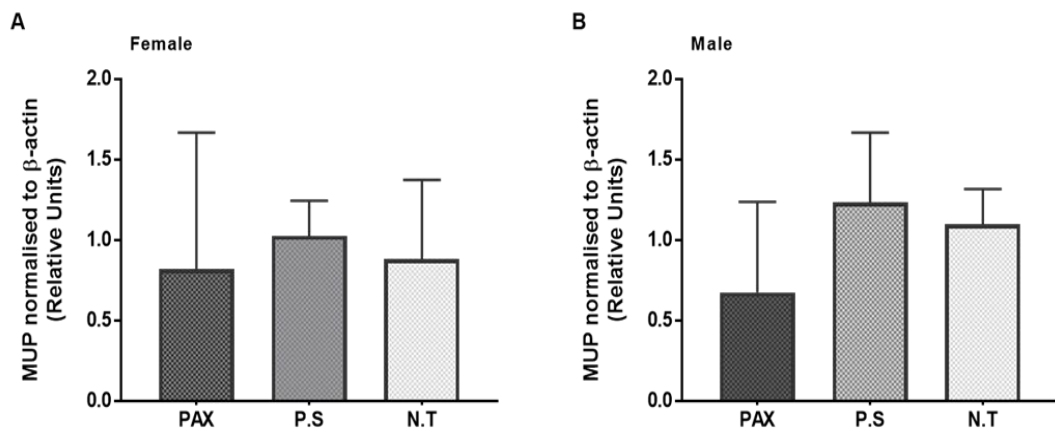
**Figure 5-12. SDS-PAGE analysis for PAX-treated female mice (FMS-PAX, gel A) and male mice (MMS-PAX, gel B). 20 $\mu$ g of protein was loaded in each lane, then separated proteins were stained using Coomassie blue stain. For both gels (A and B), Lane M, Molecular weight marker; Lane 1, PAX mouse-1; Lane 2, PAX mouse-2; Lane 3, PAX mouse-3; Lane 4, PS mouse-1; Lane 5, PS mouse-2; Lane 6, PS mouse-3.**

Further, gels were processed for Western blot analysis to evaluate MUP expression in response to PAX and its solvents (PS) (Figure 5.13). Treatment with PAX for three female mice displayed high discrepancy, where mouse number 1 and 3 presented a very low amount of MUP compare to mouse number 2 which showed a relatively high amount. For mice treated with PAX-solvent (PS), the amount of MUP was elevated and consistent. In this experiment, one untreated female mouse (NT number 3) was replaced with MUP positive control.

I



II



**Figure 5-13. Comparison of MUP expression between FMS-PAX and MMS-PAX.** Samples were treated with; PAX (n=3), PS (n=3), and N.T (n=2 for female and n=3 for male) in triplicate for western blotting analysis (I).  $\beta$ -actin in the same liver extract was used as an internal reference. Optical density reading values of the MUP protein was normalized using the expression of  $\beta$ -actin, then represented as a normalised ratio of MUP expression (II). P-value was calculated following Students' t-test by comparing treated samples with their corresponding control (N.T), for female Set (A) and male Set (B). +ve control is MUP1 recombinant protein included as a positive standard.

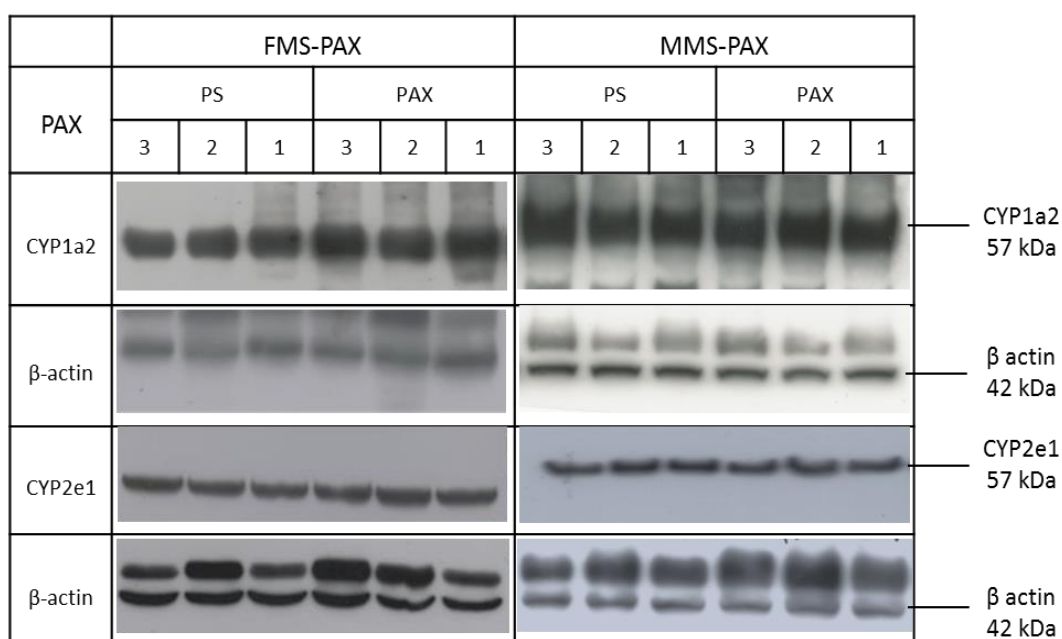
The expression of MUP in NT-female mice was also consistent and in low amount. On the other hand, male mice treated with PAX and its solvent (PS) showed greater consistency, with exception of PAX mouse 1, where the amount of MUP was unexpectedly low. However, these results highlight the occurrence of individual variation in MUP expression.

Additionally, there was no significant variation between PAX or PS -treated mice and NT, in male and female sets.

#### 5.3.4.4 Sex effect of treated mice with PAX on CYP

Similar to DOX, PAX was used to investigate whether it has the ability to modulate the expression of CYP1a2 and CYP2e1 in male and female mice (Figure 5.14). PAX exhibited no significant differential expression for either CYP1a2 or CYP2e1.

**A**





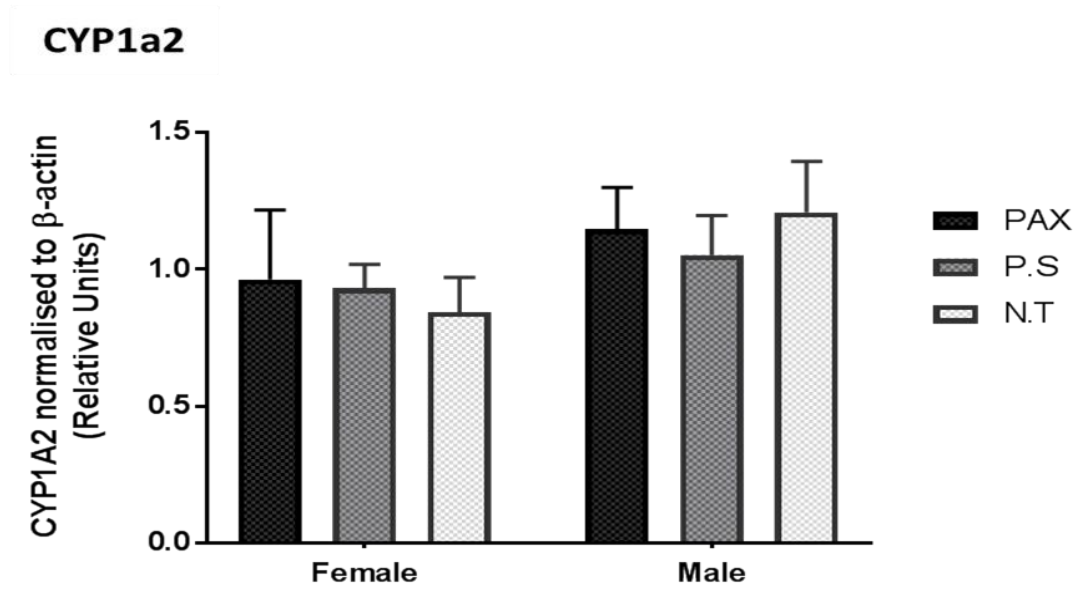
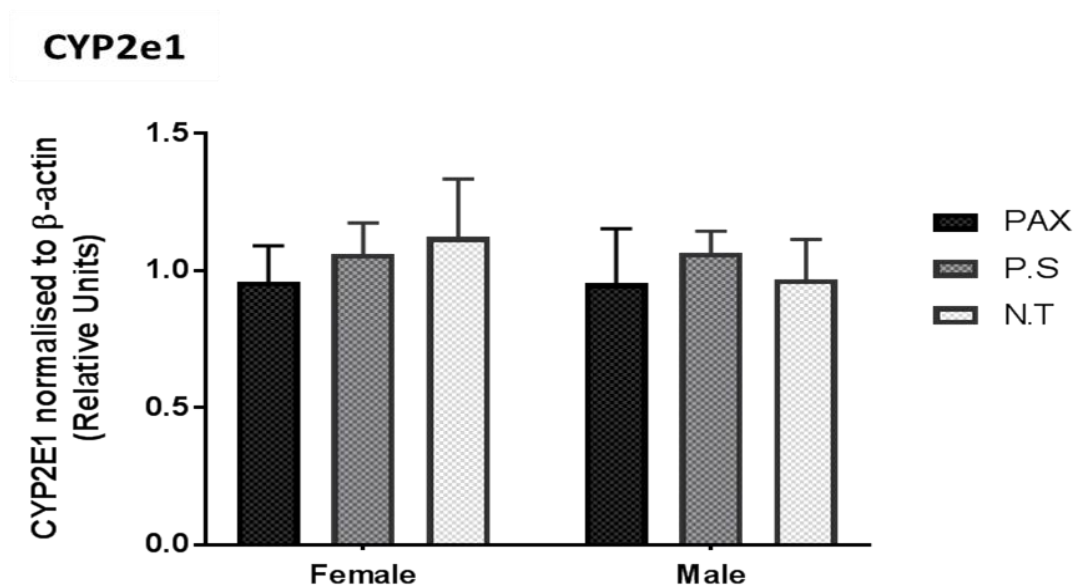
**B****C**

Figure 5-14. Comparison of CYP1a2 and CYP2e1 expressions between FMS-PAX and MMS-PAX. Samples were treated with; PAX (3 mice for each set), PS (3 mice for each set), and NT (3 mice for each set) in duplicate for western blotting analysis (A).  $\beta$ -actin in the same liver extract was used as an internal reference. Optical density reading values of the CYP protein was normalized using the expression of  $\beta$ -actin, then represented as a normalised ratio of CYP1a2 (B) and CYP2e1 (C) expressions. P-value was calculated following Students' t-test by comparing treated samples (PAX or PS) with their corresponding control (NT).

## **5.4 Discussion**

### **5.4.1 Validation MUP as a potential biomarker from quantitative proteomics datasets A and B**

Liver microsomes from datasets A and B (Chapter 4) were used in the first part of this Chapter to confirm the observed changes in MUP expression via Western blotting techniques. In spite of partial degradation in liver samples of Set A, quantitative comparison between proteomics iTRAQ ratios and Western blotting band intensities showed strong agreement and reproducible data (Table 5.2). Furthermore, statistically, significant up- and down-regulated isoforms were consistent between proteomics and Western blotting data for both datasets. Based on the supplier, the MUP1 antibody may bind other isoforms of MUP, especially MUP2, which participate 98% of amino acid sequence with MUP1. The fold changes in MUP of liver treated with DOX compared to control (7.2) is slightly higher than the relative iTRAQ ratio of MUP1 (6.27), which may prove that MUP antibody binds more than one isoforms with different affinity.

As indicated in the previous chapter, we believe that some factors have affected the results of MUP from Sets A and B. The variation in the proteomics results may have been due to one or more experimental variables; 1) using different controls since the control of Set A has not been injected with normal saline as in Set B, 2) the sex variation effect, 3) drug solvent effect, and 4) inter-individual variation.

**Table 5-2. Comparison MUP expression (Set A and B) between quantitative proteomics iTRAQ data and Western blotting data. Fold changes based on the iTRAQ ratio is representing the amount of particular MUP in treated sample to a corresponding control. Fold changes in Western blotting data is the optical density of total MUP in treated band to corresponding control band. ND; not detected, (\*) indicates significantly up-regulated, (\*\*) indicates significantly down-regulated.**

	Proteomics data						Western blotting data					
MUP isoforms	Fold Changes – iTRAQ ratio						Fold Changes – intensity ratio					
	Set A			Set B			Set A			Set B		
	5FU	CIS	DOX	5FU	PAX	TCPOBOP	5FU	CIS	DOX	5FU	PAX	TCPOBOP
MUP1	2.18*	2.47*	6.27*	1.07	1.06	1.52*	1.3*	2.3*	7.2*	1.2	0.96	2.5*
MUP2	1.81*	2.04*	2.78*	ND	ND	ND						
MUP3	1.25	1.19	2.05*	ND	ND	ND						
MUP6	1.74*	2.21*	3.34*	0.89	0.70**	1.63*						

Accordingly, we have verified the expression of MUP and CYP450 enzymes, CYP1a2, CYP2e1, and CYP3a, in new sets of male and female mice. Importantly, we have demonstrated that anti-human CYP1A2 and anti-CYP3A4 antibodies can be used to detect equivalent mouse isoforms. In the latter case, we cannot be definitive about the mouse isoforms detected as four mouse CYP3a isoforms were detected by proteomics analysis (Chapter 4, Table 4.10), including CYP3a11, which has greatest sequence homology to CYP3A4.

In order to exclude the factors of variation in drug effect on proteins, we have studied the sex-effect on each of the protein of interest alone and then this effect on the proteins upon DOX and PAX administration. In addition, we have taken the possible effect of drug solvent into consideration.

#### **5.4.2 Sex effect on protein expression of MUP, CYP1A2, CYP2E1, and CYP3A**

Major urinary proteins (MUPs) are a subfamily of proteins that descends from a superfamily called lipocalins. Our study has revealed a noteworthy sex variation in the expression of MUP in untreated males and females, having 3:1 ratio of males to females. This result is in line with previous studies that demonstrated higher male levels of MUP from both laboratory-bred strains and wild caught strains (Beynon and Hurst, 2004, Finlayson et al., 1963, Payne et al., 2001), and supports the data of a previous study that testosterone has an effect on the levels of MUP (Szoka and Paigen, 1978). However, the previous studies showed that the levels of MUP correlate to its concentration in urine, while our study has compared the levels of protein expression in the liver, which helps to avoid some factors that may be related to dilution following its expression from the liver via the bloodstream to the kidney.

Regarding hepatic CYP450s, sex variation in expression is highly important due to its major impact on drugs and xenobiotics metabolism. In general, sex dimorphism has been observed and reviewed by previous studies but remains controversial (Waxman and Holloway, 2009, Shapiro et al., 1995). The current study has investigated such dimorphism in the protein levels of three CYP450s, CYP1a2, CYP2e1, and CYP3a, which have a well-known role in cancer etiology and drugs metabolism.

Our results have shown a significant sex difference in protein expression of CYP1a2 ( $p < 0.05$ ), being higher in males than in females. This result is consistent with a recent study that demonstrated a male-bias in the CYP1a2

in murine liver microsomes using Multiple Reaction Monitoring (MRM)-MS-based quantitation technique (Hersman and Bumpus, 2014). However, a previous study (Zhang et al., 2011), contradicts our results by observing higher levels in women. This discrepancy might be attributed to use of a microarray to measure the gene expression on the mRNA level, while we have used Western blotting to measure the enzyme expression at protein level. Moreover, the tissue the authors used was a human tissue from patients who have primary liver tumours removed. Also, the liver tumour might have added confounders affecting their results, especially that CYP1A2 has been previously found to be the most affected by liver diseases (George et al., 1995b).

In regard to CYP2e1, no significant difference has been found in the levels of CYP2e1 in female mice compared to males though there was inter-individual variation in females. Our results are in agreement with a previous proteomics study, which found no sex difference in protein level using MRM-MS technique (Hersman and Bumpus, 2014). Similarly, a previous study has observed no difference in CYP2E1 mRNA and protein levels between male and female mice. However, their data did reveal a fluctuation in the CYP2e1 levels in females based on the phase of the female estrous cycle, with the highest expression of CYP2e1 at the estrous phase and lowest at metestrus (Konstandi et al., 2013) and may explain the inter-individuality we observed.

Our study has found no significant sex-related variation in the protein expression of CYP3a. These findings are consistent with those observed by another previous study, which demonstrated sex-independent and GH-unresponsive hepatic pattern in murine CYP3a11 mRNA expression, the most

homologous isoform in the mouse to human CYP3A4, an attribute verified by our use of an anti-3A4 antibody to analyse mouse samples (Cheung et al., 2006). Previous attempt to investigate the effect of 3-methylcholanthrene on mouse hepatic CYP3A expression showed that CYP3A4 antibody produced in the rat is primarily bound CYP3a11 (Lee et al., 2013a).

Furthermore, no sex dimorphism has been found in another previous study when investigating mRNA expression in either CYP3A11, CYP3A13, CYP3A25, and CYP3A57 isoforms (Renaud et al., 2011). However, in contrast to our results, a previous study indicated differential expression of the CYP3A4 protein in human liver microsomes at mRNA and protein levels (Wolbold et al., 2003). However, inter-individual variation in human CYP3A4 is highly prevalent, and 90% is attributable to genetic factors (Özdemir et al., 2000).

#### **5.4.3 Effect of DOX and PAX on MUP**

To the best of our knowledge, we are the first study to observe the acute effect of DOX on MUP and show a significant induction of protein expression in male mice. However, this induction has not been observed in female mice. Previous studies have found that other drugs, Phenobarbital (PB), 3-methylcholanthrene (3-MC) and N-nitrosomorpholine (NNM) alter MUP expression (Zgoda et al., 2006, Glückmann et al., 2007). The authors suggested that MUPs may have a role in the binding of PB and 3-MC or their oxidation products, as well as participating in their corresponding complexes excretion.

Circulating MUP is reported to have a physiological role in regulating glucose and lipid levels and markedly reduces hyperglycaemia in mouse liver (Zhou et

al., 2009). On the other side, DOX has been found to cause insulin resistance and a tremendous increase in serum glucose (Arunachalam et al., 2013), as well as accumulation of fatty acids (Hammer et al., 2010). Interestingly, DOX has caused an up-regulation of FABP in our results. Taken together, the regulatory role of MUP in glucose and lipid levels and the effect of DOX on glucose and fatty acids may be related.

DOX has been shown to activate aryl hydrocarbon receptor (AhR) (Volkova et al., 2011). 3-MC which also activates AhR (Pansoy et al., 2010), whose activation was found to increase lipids, glucose levels, and hepatic oxidative stress, has also been found to increase MUP expression (Biljes et al., 2015, Minami et al., 2008, Lee et al., 2010). Moreover, administration of NNM, which causes a decrease in glucose levels (Nehrbass et al., 1998), has shown a down-regulation in MUPs (Glückmann et al., 2007).

PAX effect has also been investigated on MUP protein expression in males and females, with a remarkable inter-individual variation in the treated mice. Overall, our results have revealed no significant effect of PAX on both males and females MUP expression.

#### **5.4.4 Effect of DOX and PAX on CYP1A2 and CYP2E1**

DOX has been reported to cause cardiotoxicity which is suggested to be associated with CYP450 enzymes. Previous studies have investigated such association at mRNA levels. However, the effect of DOX has not been extensively studied at the protein level. Our study demonstrated that DOX (10 mg/kg, IP, 24 hours) had no significant effect on CYP1a2 and CYP2e1 expression in either sex. A previous study (Zordoky et al., 2011) also found

no altered regulation in hepatic CYP2e1 expression at both gene and protein level by DOX (15 mg/kg, IP, 24 hours) using RT-PCR and Western blotting techniques in male mice. In contrast to our study, a previous study has observed an induction in both CYP1A2 and CYP2E1 mRNA expressions in a human cardiac cell line, H9c2 (Zordoky and El-Kadi, 2008). Likewise, PAX had no significant effect on CYP1a2 and CYP2e1 in our results. The comparison between proteomics data (Chapter 4) and Western blotting data (Chapter 5) regarding CYP1a2 and CYP2e1 is illustrated in Table 5-3.

**Table 5-3. Comparison CYP1a2 and CYP2e1 expressions between quantitative proteomics iTRAQ data (Set A and Set B) and western blotting data (FMS and MMS). Fold changes based on the iTRAQ ratio is representing the amount of particular CYP isoform in treated sample to a corresponding control. Fold changes in Western blotting data is the optical density of total CYP isoform in treated band to corresponding control band. (\*) indicates significantly altered protein.**

	Proteomics data		Western blotting data							
CYP isoform	Fold Changes – iTRAQ ratio		Fold Changes – intensity ratio							
	Set A	Set B	FMS				MMS			
	DOX	PAX	DOX	NS	PAX	PS	DOX	NS	PAX	PS
CYP1a2	1.09	0.88	1.03	0.98	1.27	0.94	1.02	0.97	0.83	0.98
CYP2e1	0.69*	1.07	0.83	0.92	1.07	0.89	1.11	0.96	0.92	1.09

Results from either DOX (Set A, male mice) or PAX (Set B, female mice) of proteomics experiments were in agreement with data obtained from Western blotting analysis. Moreover, the latter technique involved the solvent of each drug to ensure that the observed changes were because of drugs, not their solvents. However, an exception is CYP2e1 in response to DOX treatment, which was significantly down-regulated in proteomics analysis but not in Western blotting analysis. Even though, the expression of DOX in FMS was



slightly reduced (0.83-fold change) but not in MMS. This result might highlight the short duration of treatment, alongside whether CYP2e1 antibody has cross-reactivity with other mouse CYP isoforms.

In conclusion, we suggest that MUP altered-regulation only occurs in response to a physiological function alteration, and we can propose MUP as a candidate biomarker for hepatotoxicity in male mice. Our study has presented an investigation assay that will enable further exploration of the impact of different drugs on MUP and some CYP450, taking sex effects into consideration. We suggest further investigation in regard to DOX and PAX effect, over a longer time course, on MUP to verify its function in response to drugs, as well as on CYP1a2 and CYP2e1 enzymes due to their significant role in metabolizing drugs and cancer etiology.

## Chapter Six: Investigating the influence of DOX and PAX on the activity of cytochrome P450s in mouse liver microsomes using VIVID assay

## **6 Investigating the influence of Doxorubicin and Paclitaxel on the activity of cytochrome P450s in mouse liver microsomes using VIVID**

### **6.1 Introduction:**

As the mRNA levels vary, the proteins also vary, and so does the activity of these proteins. The post-translational modifications that the proteins might undergo to regulate their activity, localization or interaction with other cellular molecules, result in protein functional diversity and this has brought meaningful insights toward studying the activity of the enzyme in addition to its gene and protein expressions (Karve and Cheema, 2011). Moreover, changes in protein expression do not necessarily reflect changes in activity, as the protein expression is more stable and easily analyzed compared to activity assessment (Myers et al., 2012). Understanding the changes in the activity of proteins in addition to their expression will accelerate the process of understanding the mechanistic basis of human disease and developing clinically effective drugs.

As described earlier, the mechanism by which CYP450s exert their function in phase I metabolism revolves around using molecular oxygen ( $O_2$ ) to give the substrate (i.e. drug) an oxygen atom through what is called mono-oxygenase cycle, which requires some cofactors, including NADPH oxidoreductase enzyme and cytochrome<sub>b5</sub> and specific conditions to be accomplished (Rang et al., 2014). Therefore, assessing the enzyme activity *in vitro* is considered a challenge as all the components of the CYP kinetic cycle (Figure 1.3) should be available in appropriate amounts, in addition to the pH and temperature

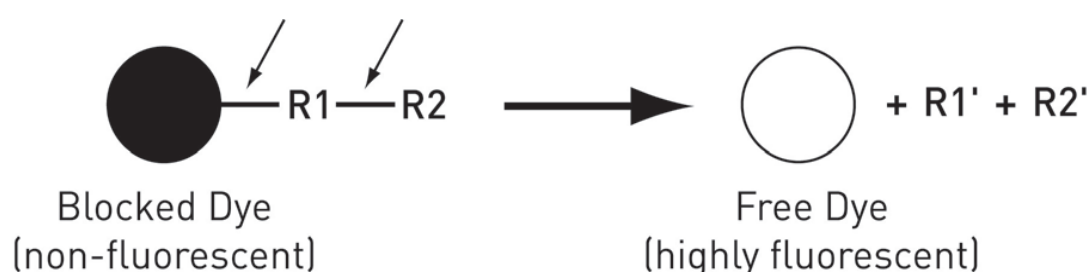
that should be optimized carefully in order to obtain the maximum activity of the enzyme. Also, care is required during the mechanical destruction of the cells for proteins extraction, so as to maintain the enzyme active (Wienkers and Heath, 2005, van Eunen and Bakker, 2014, Scopes, 2002).

CYP450 enzyme activity is evaluated based on the rate of forming a marker metabolites, which can be detected by one of these approaches; luminescence, fluorescence, radioactivity, photometric or mass spectrometric assays (Paul et al., 2012). The fluorescence method is the most used method for CYP450 activity evaluation and is based on the incubation of an appropriate non-fluorescent substrate with a specific CYP450 enzyme converting it into a fluorescent metabolite. The rate of changes in fluorescence intensity represents the quantitative measure of the fluorescent metabolite formed, hence the measure of enzyme activity.

Each method has its own advantages and limitations. For instance, photometric and fluorometric assays are considered superior over the radiometric that they are continuous, accurate with high throughput, and less hazard and cost (Paul et al., 2012, Liao et al., 2015). Meanwhile, the fluorometric assay is considered more sensitive and reproducible than photometric and lower amounts of both substrate and enzyme are needed, accordingly (Chen et al., 2005). Nevertheless, photometric and fluorometric assays are not applicable in the case of non-chromogenic or non-fluorogenic substrates which are still a challenge for chemists to synthesize (Paul et al., 2012). On the other hand, the mass spectrometric assay is used for those substrates that lack chromophore or fluorophore, but unfortunately, it is a

discontinuous method and requires high costly equipment (Paul et al., 2012, Wu et al., 1997).

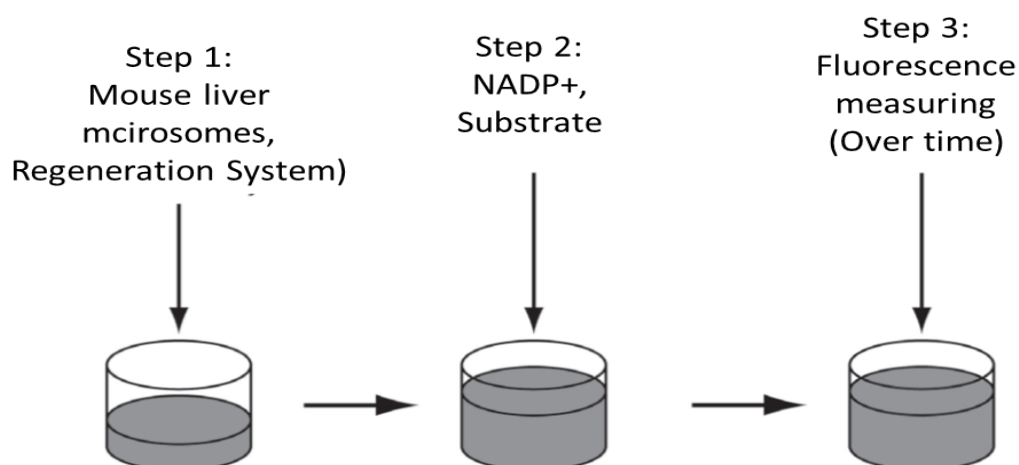
The VIVID assay is sensitive, homogeneous, cost effective fluorometric method with a high throughput (Trubetskoy et al., 2005b). VIVID fluorogenic substrates are metabolised by CYP450s into highly fluorescent products in the visible light spectrum with minimal interference from background fluorescence (Figure 6.1), which reflects the concentration of the metabolite and thus, enzyme activity (Trubetskoy et al., 2005b).



**Figure 6-1. Schematic representation of metabolizing fluorogenic VIVID substrate by a cytochrome P450 enzyme from “blocked” dye into a fluorescent metabolite. The arrows indicate the two potential P450 cleavage sites. Oxidative cleavage at either site releases the highly fluorescent product.**

VIVID assay was originally designed for evaluating the power of various compounds in inhibiting human CYP450 isoforms; by incubating microsomes from baculovirus-infected cells co-expressing CYP450 isoform (i.e. baculosome CYP450) contains reductase cofactors (NADPH-cytochrome P450 reductase and cytochrome<sub>b5</sub>) provided by Invitrogen with an experimental inhibitor, then measure the inhibition percent for this compound. However, we modified this assay by replacing the baculosome CYP450 enzyme with mouse liver microsomes to be able to measure the activity of particular microsomal CYP450 enzyme.

In the modified workflow, a master pre-mix is prepared by mixing a defined amount of liver microsomes proteins with NADPH regeneration system (glucose-6-phosphate and glucose-6-phosphate dehydrogenase), which converts NADP<sup>+</sup> into NADPH, and thus required to start the CYP450 reaction. Then the pre-mix is mixed with VIVID fluorogenic substrate and as demonstrated in Figure 6.2. The feature of using the regeneration system is to minimise the fluorescence background of NADPH, which is the main component of the mono-oxygenase cycle.



**Figure 6-2. A schematic representation of a kinetic Vivid® CYP450 Assay. In the first step, a master pre-mix is prepared by combining liver microsomes with regeneration system (consisting of glucose-6-phosphate and glucose-6-phosphate dehydrogenase). Next, the master pre-mix is mixed in step 2 with the corresponding VIVID substrate and NADP<sup>+</sup> to initiate the reaction. Finally, in step 3, fluorescence readings are continuously measured over time for kinetics-related calculations.**

Two examples of VIVID substrates are ethoxymethoxy-3-cyanocoumarin (EOMCC) and 7-benzyloxymethoxy-3-cyanocoumarin (BOMCC). They possess a high aqueous solubility, which allows using a lower concentration of the organic solvents such as acetonitrile and DMSO (Trubetskoy et al., 2005b). This feature is of a high importance as the organic solvents are well

known to affect the metabolism by CYP450 (Easterbrook et al., 2001). A further advantage of the VIVID assay substrates is their emission and excitation wavelengths. As opposed to many other fluorophores, the emitted wavelengths lie further away from the wavelengths of NADPH. Although, the VIVID substrates EOMCC and BOMCC are used to assay CYP1A2 and CYP3A4, respectively, they have limited specificity, which is an inherent challenge of analysing CYPs. For example, BOMCC has cross-reactivity with other isoforms including, CYP2B6, CYP2C9, and CYP3A5, and EMOCC towards CYP2C19, CYP2D6, and CYP2E1.

The VIVID assay has been applied to assess the capacity of several chemical and herbal compounds in inhibiting CYP450 enzymes activity by previous studies. Trubetskoy *et al* have investigated the effect of a CYP inducer, rifampicin, on CYP3A4 activity in DPX-2 cell line (Trubetskoy et al., 2005a), Marks *et al* investigated the metabolism and inhibition of CYP2E1 by known CYP2E1 inhibitors such as propofol and ketoconazole (Marks et al., 2002) Another study evaluated the effect of short term and long term exposure of an herbal supplement on oral bioavailability of nevirapine (Minocha et al., 2011) using the VIVID assay. Furthermore, novel CYP450 inhibitors have been evaluated by our group using the high throughput VIVID assay (Sellars et al., 2016). The VIVID assay has also been applied on the human liver microsomes to assess the effect of some haplotypes in cytochrome P450 oxidoreductase (CYPOR) gene on the activity of CYP3A4 (Moutinho et al., 2012). Hence, the VIVID assay provides a robust tool to understand the mechanism behind drug-drug interaction, drug resistance, drug response and toxicity. Furthermore, it

is considered a high throughput and sensitive step in drug development studies.

Liver microsomes are the most widely used amongst *in vitro* technologies in metabolizing enzymes activity profiling (Fasinu et al., 2012), but has not previously been studied using the VIVID assay.

In order to measure enzyme activity from liver microsomes, VIVID assays were slightly modified. Microsomal proteins were enriched via centrifugation and ultracentrifugation to get rid of the majority of proteins that might interfere with the enzyme activity assessment.

#### **6.1.1 Aims of the chapter**

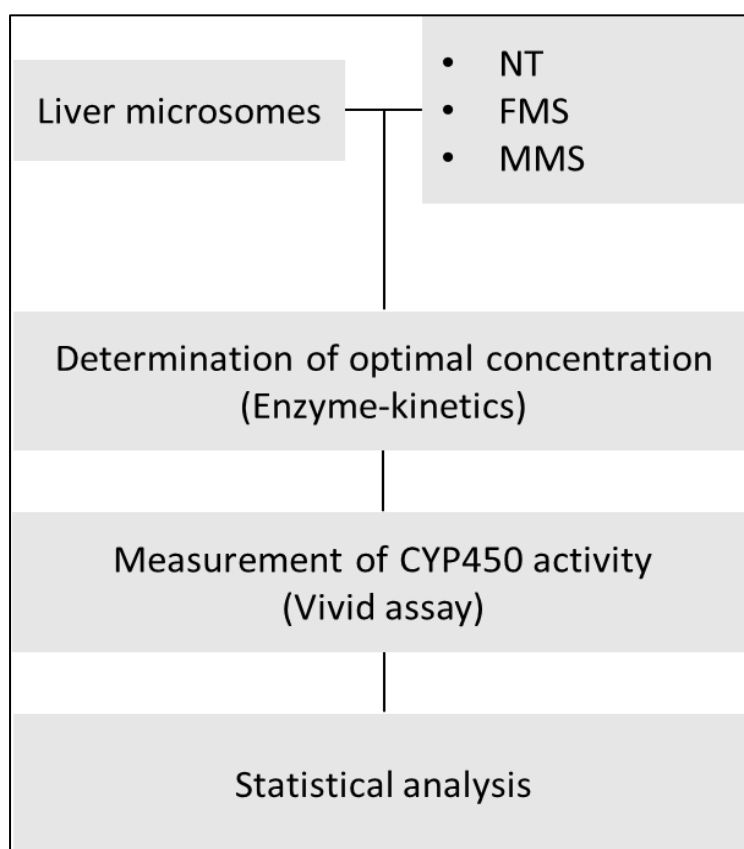
Since the alteration in expression does not necessarily reflect alteration in enzyme activity and as we are using liver microsomes extracted from mouse liver, the first aims of this chapter is:

- a. To determine if the VIVID assay could be used to measure CYP1a2 and CYP3a activity in mouse liver microsomes
- b. To test and optimise the ability to use VIVID substrates in measuring the endogenous CYP450s activities.
- c. To evaluate and correlate the observed changes, particularly in CYP450 isoforms (CYP1a2 and 3a), in previous chapters (Chapter 4 and 5) by using the VIVID assay



## 6.2 Material and Method

Materials and methods used in the VIVID assay were described in detail in Sections 2.1.3 and 2.2.1, respectively. Samples of liver microsomes from female (FMS) and male (MMS) mice treated with DOX and PAX, in addition to their solvents prepared in the previous chapter (section 5.2.1) were used for the VIVID assays (Figure 6.3).



**Figure 6-3. Flow diagram showing the steps of measuring the activity of CYP450 enzymes using VIVID assay. No treatment – NT, treated female (FMS) and male (MMS) mice sets, were prepared in Chapter 5. Then the optimal amount of microsomal proteins was determined and used further for VIVID activity assay.**

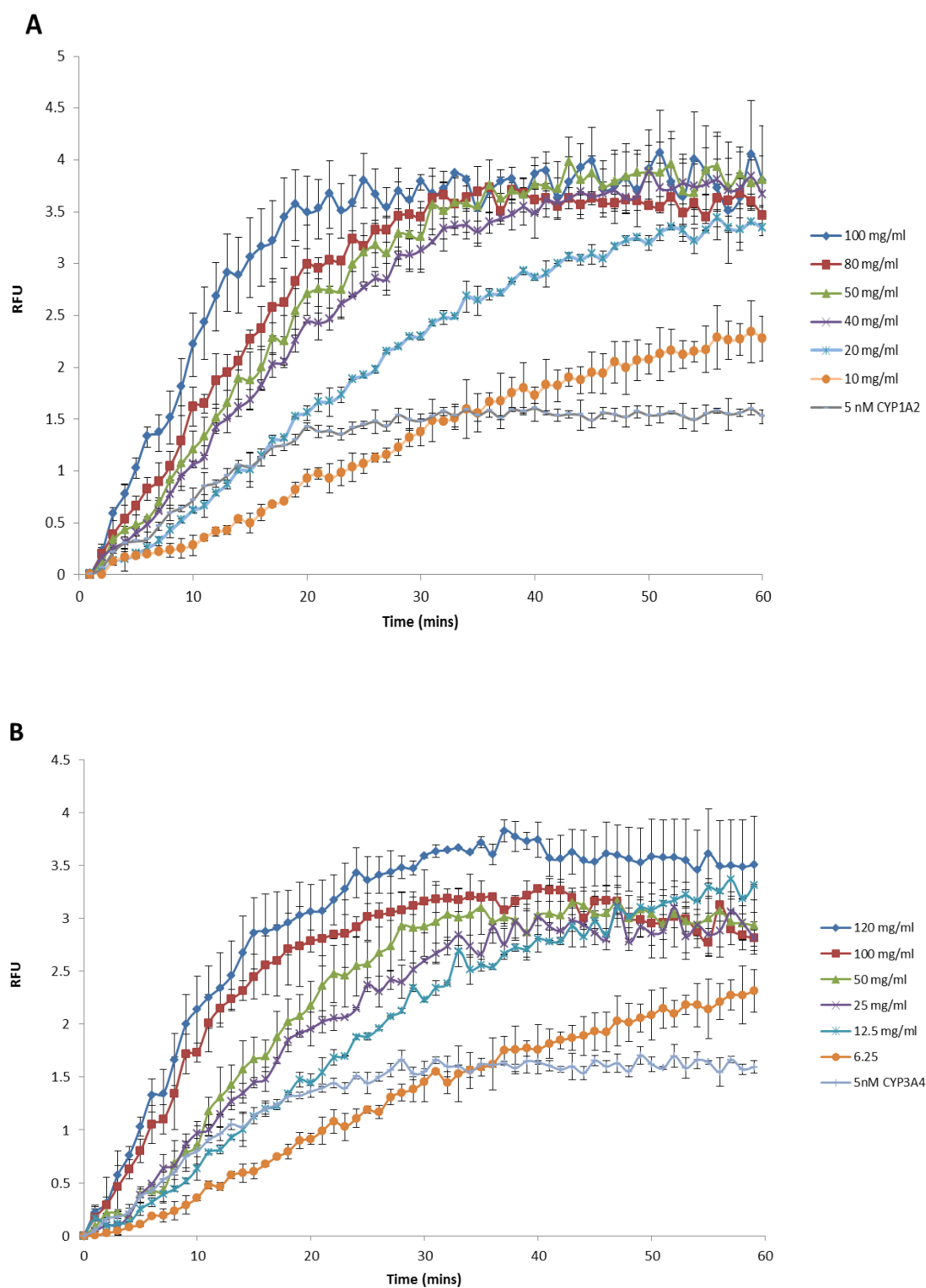
## **6.3 Results**

### **6.3.1 Determine whether the VIVID assays detect and enable the measurement of mouse liver CYPs**

#### **6.3.1.1 Optimisation of mouse liver microsomes protein concentration**

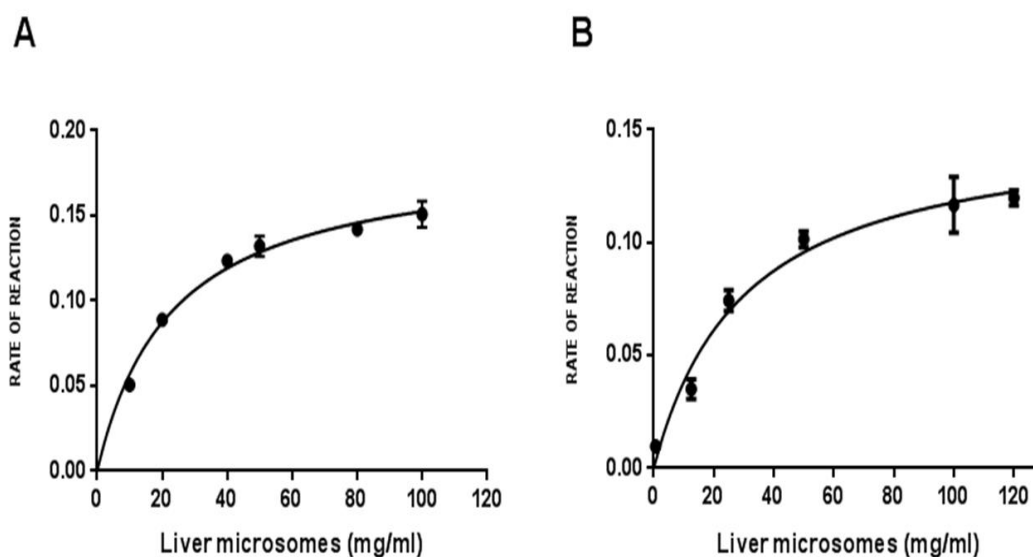
Normal and treated mouse liver microsomes from both sexes were used to evaluate the activity of CYP1a2 and CYP3a. initially, recommended concentration of substrate (3 $\mu$ M EOMCC for CYP1a2 or 5 $\mu$ M BOMCC for CYP3a) was incubated with different amount of microsomal proteins (6.25 to 120 mg/ml); thus normal untreated (NT) male samples (three mice) were used to determine the best concentration of microsomal proteins that can be used further for measuring the activity of CYP1a2 and CYP3a (Figure 6.4).

Keeping the substrates concentrations constant in each reaction and increasing the concentration of the microsomal protein displayed a corresponding increase in enzyme activity. The rate (enzyme activity) of metabolite formation in the reaction of CYP1a2/CYP1A2 with the fluorogenic substrate (EOMCC) started to plateau at concentrations 40 mg/ml and above (Figure 6.4A). The rate of CYP3a/CYP3A4 activity exhibited similar properties but at different concentration (25 mg/ml) of liver microsomal proteins (Figure 6.4B). The term “activity” will be expressed by rate, which is computed throughout this Chapter by taking the slope of relative fluorescence units (RFU) per minute within 10 – 20 minutes.



**Figure 6-4. Determination of the optimal concentration of mouse liver microsomes. A** serial dilution of microsomes was incubated with either 3 $\mu$ M EOMCC substrate for CYP1a2 (A) or 5 $\mu$ M BOMCC substrate for CYP3a (B). The VIVID assay was carried out twice and readings were taken every minute for 60 minutes as relative fluorescence unit (RFU). Recombinant human CYP1A2 (5nM) and CYP3A4 (5nM) baculosomes were included as standards

The maximum reaction velocity (Apparent  $V_{\max}$ ) and Michaelis-Menten constant ( $K_m$ ), which would be the half of the concentration that would give  $V_{\max}$  were determined using the Michaelis-Menten's equation (Figure 6.5, Table 6.1).



**Figure 6-5. The rates (RFU/min) of enzyme activity for CYP1a2 (A) and CYP3a (B) were calculated by taking the slope at 10-20 minutes (n=3), and then the rate was plotted versus corresponding protein concentration of liver microsomes to calculate  $V_{\max}$  and  $K_m$ .**

For both activity assays, 25mg/ml was determined to be the optimal concentration to be used for mouse CYP activity assays.

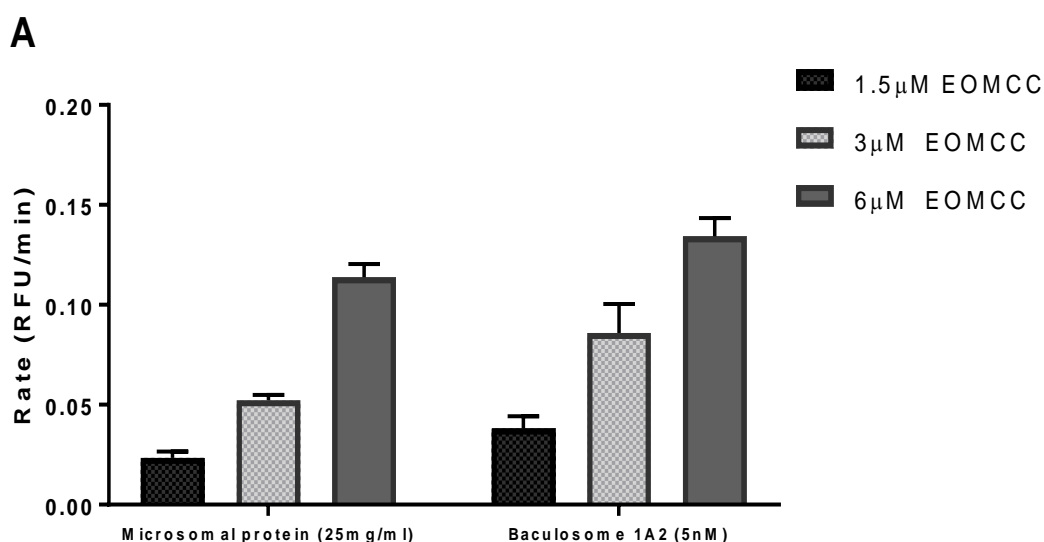
**Table 6-1. The kinetic parameters  $V_{\max}$  and  $K_m$  were calculated. GraphPad Prism was used to calculate best-fit values based on Michaelis-Menten equation**

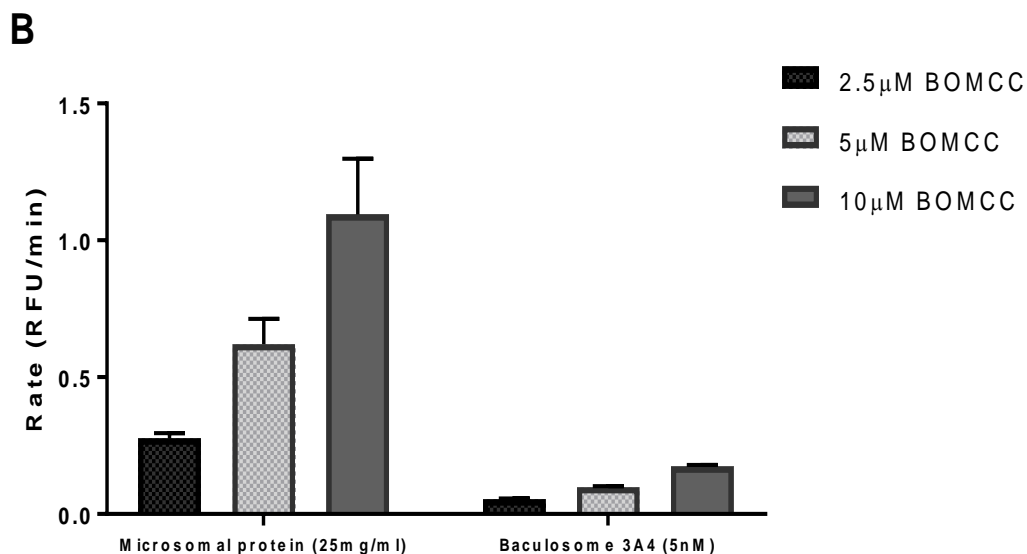
Michaelis-Menten parameters	CYP1a2	CYP3a
$V_{\max}$ (mmol/min/mmol)	$0.187 \pm 0.006$	$0.1533 \pm 0.01$
$K_m$ (mg/ml)	$22.99 \pm 2.31$	$30.12 \pm 5.63$

### 6.3.1.2 Optimisation of the substrate concentration

The optimized concentration of liver microsomes (25mg/ml) was used in order to determine the optimal concentration of EOMCC and BOMCC substrates and compare the rate of reactions to human CYP baculosome standards (Figure 6.6A and B). Each substrate, EOMCC (6 to 1.5 $\mu$ M) and BOMCC (10 to 2.5 $\mu$ M), was titrated with a constant amount of mouse liver microsome (25mg/ml).

In both cases, there was a linear increase in CYP activity with increasing substrate concentrations. Based on the kinetics calculation (Table 6.1) for the male set, the concentration 25mg/ml compared well to the recombinant human CYP1A2 or CYP3A4. 3 $\mu$ M and 10 $\mu$ M of substrate EOMCC and BOMCC, respectively, were chosen for screening activity assays using 25mg/ml of mouse liver microsome proteins. These optimised concentrations from male liver microsomes were used as well for female treated and treated samples.





**Figure 6-6. Determination of the rate of CYP1a2 and 3a from male mouse microsomal protein (25mg/ml) and baculosome CYP1A2 and CYP3A4 using different concentrations of EOMCC substrate for CYP1a2/CYP1A2 activity (A) and BOMCC for CYP3a/CYP3A4 activity.**

Next, to show the best concentration of substrate that can detect a minimal activity for CYP450, Apparent  $V_{\max}$  and Michaelis-Menten constant ( $K_m$ ), were calculated for optimised amount of microsomal proteins (25 mg/ml) using serial dilution of BOMCC and EOMCC substrates (Table 6.2).

**Table 6-2. The kinetic parameters  $V_{\max}$  and  $K_m$  were calculated based on serial dilution of substrates. GraphPad Prism was used to calculate best-fit values based on Michaelis-Menten equation**

Source of CYP450	Michaelis-Menten parameters	EOMCC	BOMCC
<b>Baculosome CYP450 (5nM)</b>	$V_{\max}$ (mmol/min/mmol)	$0.086 \pm 0.002$	$0.1533 \pm 0.01$
	$K_m$ ( $\mu$ M)	$3.8 \pm 0.34$	$0.34 \pm 0.001$
<b>Microsomal proteins (25mg/ml)</b>	$V_{\max}$ (mmol/min/mmol)	$0.072 \pm 0.004$	$1.58 \pm 0.11$
	$K_m$ ( $\mu$ M)	$3.60 \pm 0.28$	$5.4 \pm 0.18$

### 6.3.2 Determination of sex-specific CYP1a2 and CYP3a activity

In the present work, the activity of CYP1a2 and CYP3a isoforms in mouse liver over both sexes was determined. Three of untreated male and female mice were used to evaluate the variation (Figure 6.7).

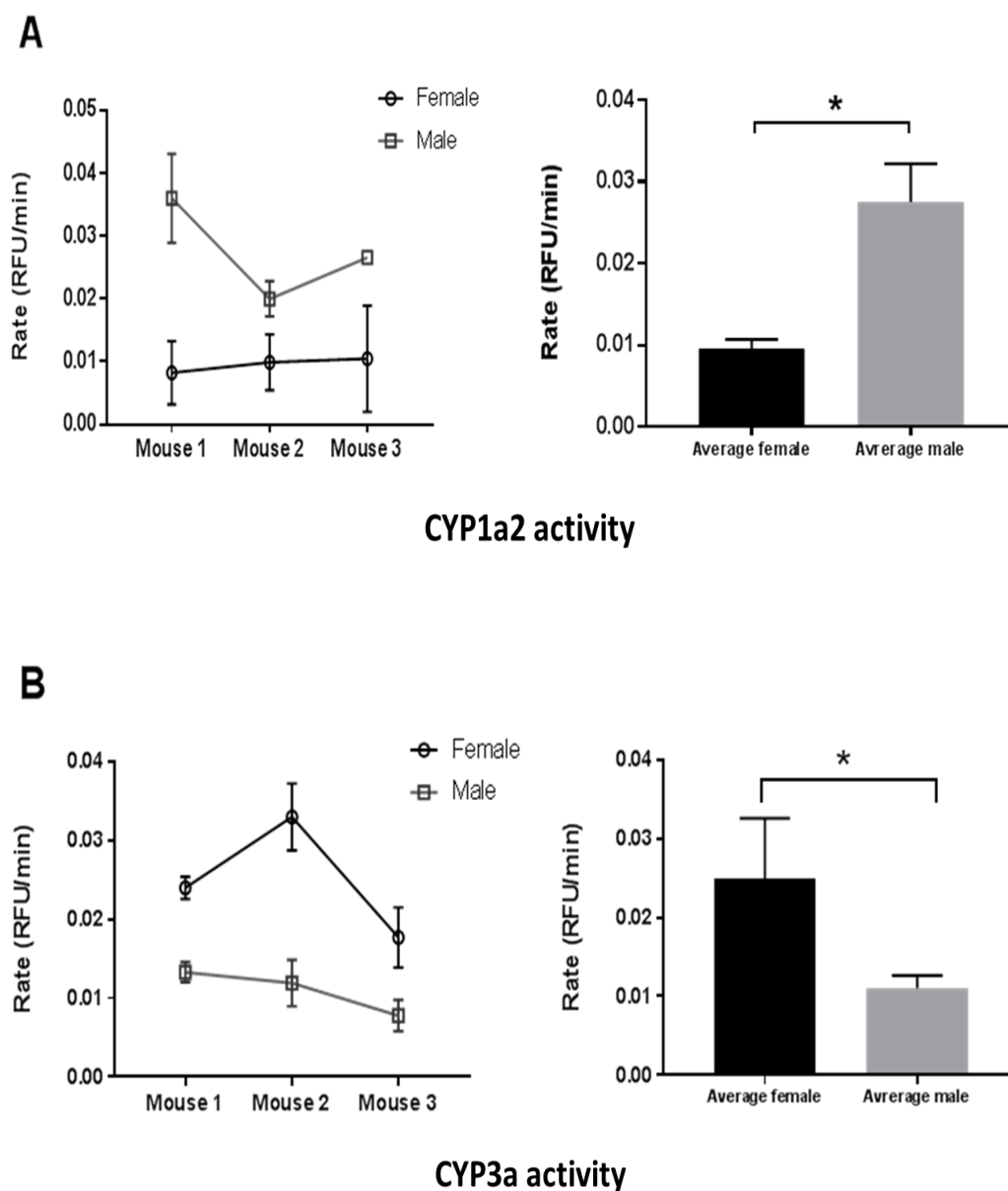


Figure 6-7. Comparison of the activity rates of untreated liver microsomal proteins from male (n=3) and female (n=3) mice in duplicate. A total of 25mg/ml was incubated with 3 $\mu$ M EOMCC substrate (A) for CYP1a2 activity and 10 $\mu$ M BOMCC substrate (B) for CYP3a activity.

By using 25mg/ml of liver microsomes from untreated male and female samples, the rate of activity of CYP1a2 was significantly higher (p-value, 0.0185) in male mice compared to female mice. The opposite was observed for CYP3a activity, where microsomal livers from female mice showed higher (p-value, 0.0427) level of activity than male mice

### **6.3.3 Measurement of CYP activity in treated male and female sets**

In a similar, but expanded the experiment to the sex-specific study, 25mg/ml of mouse liver microsome proteins from anti-cancer drug treated mice (FMS and MMS), were analysed in the VIVID assay. The activity of CYP1a2 has decreased significantly to about 50% upon administration of PAX (p-value, 0.034) and PS (p-value, 0.049) compared to untreated controls in male mice, whereas no significant effect has been observed in females (Figure 6.8). On the other hand, DOX has increased the activity of CYP1a2 in both male (p-value, 0.414) and female (p-value, 0.095) mice but not to a significant level (Figure 6.8).

Regarding CYP3a, there was a significant increase (p-value, 0.0068) in the activity of CYP3a in response to DOX in females. This inducing effect has been also observed in males with DOX; however, there was notable inter-individual variation (Figure 6.9).



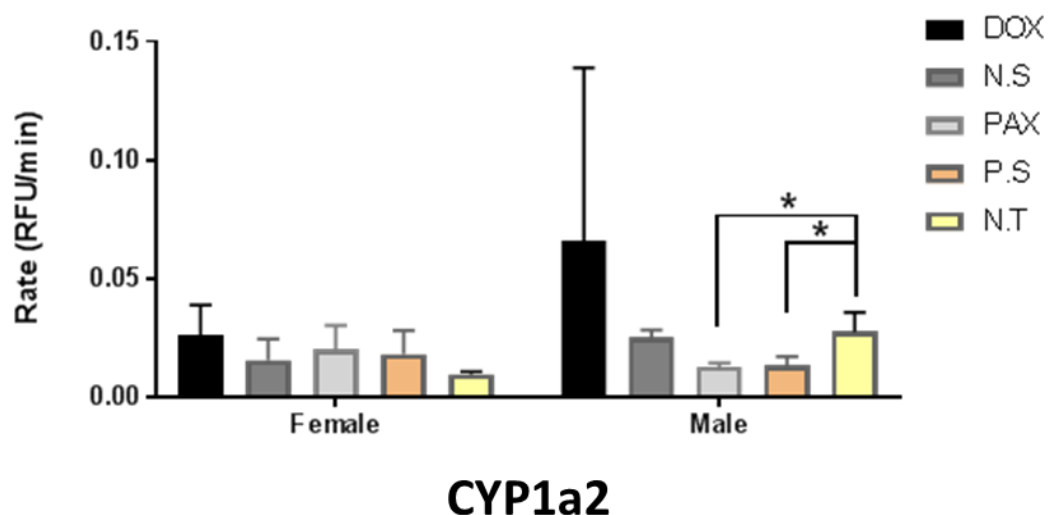


Figure 6-8. The effect of drugs and their solvents on CYP1a2 activity rate for female and male liver microsomes. The rates were calculated using the slope for 10-20 minutes. All treatments from both sexes had three mice (except NS in female had two mice). A total of 25mg/ml of each sample was incubated with 3 $\mu$ M EOMCC substrate. Each bar represents the average rate in triplicate with standard deviation.

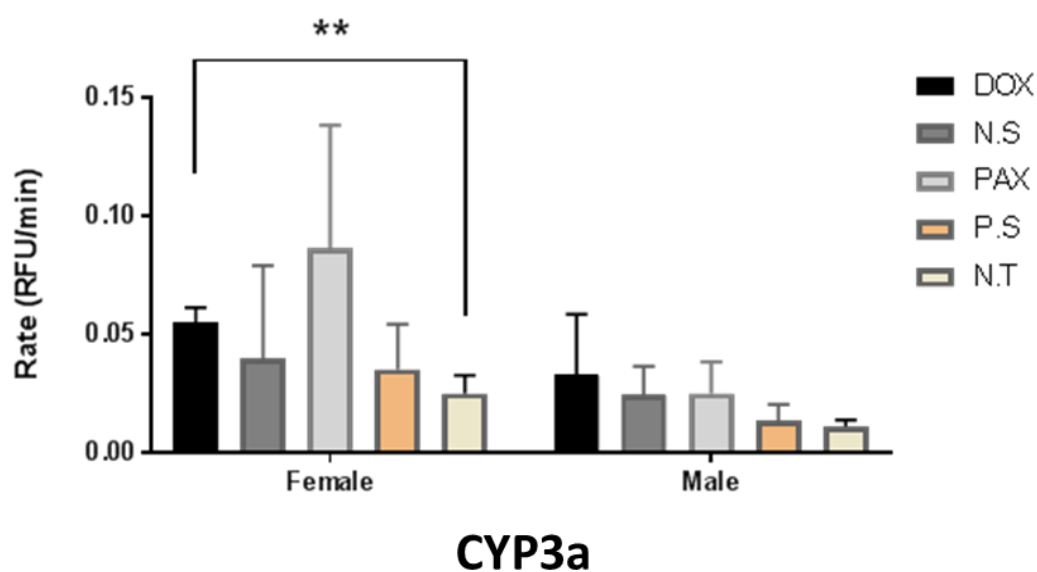


Figure 6-9. The effect of drugs and their solvents on CYP3a activity rate for female and male liver microsomes. The rates were calculated using the slope for 10-20 minutes. All treatments from both sexes had three mice (except N.S in female had two mice). A total of 25mg/ml of each sample was incubated with 10 $\mu$ M BOMCC substrate. Each bar represents the average rate in triplicate with standard deviation.

## 6.4 DISCUSSION

In this chapter, we aimed initially to establish a robust workflow that can be employed for measuring the activity of CYP450 from the biological complex mixture (i.e. liver microsomes) based on fluorescence assay. Then, we investigated and correlated (with previous results) the sex effect, as well as the anticancer drugs (DOX and PAX) effect on expression and activity of the mouse equivalents of CYP1a2 and CYP3a which play a major role in drugs metabolism and cancer susceptibility in humans.

In the first instance, the VIVID assay was successfully applied to mouse liver microsome models, which had not previously been demonstrated. Comparing the kinetics parameters ( $K_m$  and  $V_{max}$ ) for mouse CYP1a2 and CYP3a to human baculosome CYP1A2 and CYP3A4 exhibited (Table 6.2) a very close of maximum rate of reactions ( $V_{max}$ ) between human and mouse CYP1A2, indicating a high activity of mouse CYP1a2 by using 25 mg/ml of proteins that was equal to 5nM of baculosome CYP1A2. Using anti-CYP3A4 antibody for mouse liver microsomes measured the activity of multiple mouse CYP3a family members (CYP3a11, CYP3a16, CYP3a25 and CYP3a41), identified by proteomics (Chapter 4, Table 4.8). Because of the promiscuous nature of CYPs, activity from other mouse CYPs may also contribute to the observed activity.

### 6.4.1 Sex variation in CYP1a2 and CYP3a activity

In our results, the activity of CYP1a2 has been higher in untreated males than in females. This finding supports our results in the previous chapter, which revealed a significantly higher expression of CYP1a2 in untreated males

compared to females. Consistent with our findings, the previous study has found lower clearance of Clozapine, which is mainly metabolised by CYP1A2 in women (Banov et al., 1993). Moreover, Gunes *et al* have found a higher activity of CYP1A2 in males than in females in Turkish population when investigating the metabolism of caffeine, a major substrate for CYP1A2 (Gunes et al., 2009). Furthermore, and consistent with our study an *in vitro* study has demonstrated a higher metabolism of phenacetin O-deethylation, which is a marker for CYP1a2 activity, in the liver microsomes of the male mice than in females (Löfgren et al., 2004).

In regard to CYP3a, our results have revealed a significantly higher activity in females compared to males. Correspondingly, proteomics data from Chapter 4, resulted in the identification of five CYP450 isoforms (CYP3a11, CYP3a13, CYP3a16, CYP3a25, CYP3a41) out from six isoforms, which are believed to be homologue to human CYP3A4. Moreover, two of them (CYP3a16 and CYP3a41) have not been detected in Set A (Male mice), indicating them female-predominant isoforms that may a possible role of inducing the activity of CYP3a in female mice. Furthermore, the other possible isoforms rather than CYP3a that can metabolise BOMCC substrate were not detected in proteomics data of Sets A and B, indicating low amount expressed in mouse liver of both sexes.

However, our finding is in line with the previous study that found higher N-dealkylation of verapamil by CYP3A4 in human females (Wolbold et al., 2003). Moreover, an *in vitro* study (Schmidt et al., 2001) has demonstrated a higher activity of CYP3A4 in women when comparing the activity of the enzyme in 10 male and 10 female human liver microsomes using high-sensitive HPLC/MS

and -UV detection methods. Also, Parkinson *et al* have found that the activity of CYP3A4 in human liver microsomes in females is twice as high as in men, which is consistent with our results (Parkinson *et al.*, 2004).

The sex difference in the expression of CYP3A might be attributed to the pregnancy-associated progesterone hormone, which is a ligand for the nuclear receptor, pregnane X receptor (PXR) (Timsit and Negishi, 2007), whose activation is known to induce CYP3A expression (Harmsen *et al.*, 2007). Moreover, it has been found that growth hormone (GH) regulates the expression of CYP3A through enhancing the binding of the transcriptional factors to the CYP3A promoter, inducing its expression (Li *et al.*, 2015a). Here, it is worth mentioning that the GH is known to be pulsatile in male mice while it is constant in females (Waxman and Holloway, 2009), which results in sex difference in CYP3a4 expression. Such findings have been supported by another study (Dhir *et al.*, 2006) which has revealed an up-regulation of CYP3A4 mRNA expression with constant treatment with GH, whereas pulsatile GH treatment has shown a suppression of CYP3A4 mRNA expression in primary human hepatocytes.

#### **6.4.2 Effect of PAX and DOX on CYP1A2**

Regarding the activity of CYP1A2 in response to PAX, our results have shown a significantly reduced activity of CYP1A2 in PAX treated compared to the untreated mice in males but not in females. However, PS (CrEL, DMSO) has also shown a significant decrease in CYP1A2 activity and an approximately similar activity compared to PAX. This observation leads us to the assumption that the decrease in activity was due to the solvent rather than the drug

exposure. In agreement with our results, DMSO with the concentration 2% has been found to have an inhibitory effect on CYP1A2-mediated phenacetin O-deethylation (Nirogi et al., 2011). Likewise, similar inhibition has been observed when investigating the effect of DMSO (1%) using caffeine N3-demethylation as a probe reaction, which has revealed 30% decrease in CYP1A2 activity (Hickman et al., 1998). In good agreement, as a part of the previous study conducted in our lab to evaluate the inhibitory effect of novel CYP450 inhibitors, different solvents on CYP1A2 and CYP3A4 were evaluated, among them, 1% of DMSO has shown 28% inhibition in CYP1A2 activity. So we suggest that the effect of DMSO on CYP1A2 may occur at the protein level rather than the activity, as we also observed a decrease in protein expression of CYP1a2 by Western blot (Chapter 5) in response to DMSO. In addition, PAX has exhibited a decrease in CYP1a2 protein level by proteomic profiling (iTRAQ ratio = 0.88) (Chapter 4).

The sex difference that was observed by PS in males, but not in females, might be attributed to the higher levels of CYP1a2 expressed in males, so the inhibitory effect was more readily observed.

DOX has increased CYP1a2, however high inter-individual variation affected the interpretation, making the difference insignificant. To the best of our knowledge, we are the first to study that investigates the effect of DOX on the activity of CYP1a2 in liver.

#### **6.4.3 Effect of PAX and DOX on CYP3a**

PAX increased the activity of CYP3a in both males and females; however, this increase was not significant due to the high inter-individual variation. DOX-

treated mice showed a significant increase in the activity of CYP3a in females with no significant effect for the DOX solvent (NS). The effect of acute DOX administration on CYP3A4 activity has not been extensively studied, however, CYP3A4 mRNA has been found to be up-regulated in DOX-resistant MCF7 cell line (AbuHammad and Zihlif, 2013).

DOX is a major substrate of CYP3A4 (Rose, 2005) suggesting that mRNA expression of CYP3A4 may be induced in response to DOX administration to accelerate its metabolism and clearance, and thus develop DOX resistance. The mechanism by which CYP3A4 is induced in response to DOX has been proposed (Goldstein et al., 2012), in which DOX administration led to phosphorylation, and therefore activation of p53 in human liver cells. Such activation resulted in inducing CYP3A4 at both gene and activity levels.

As for PAX and CYP1a2, observing sex difference in the effect of DOX on CYP3a can be explained by having higher levels of the enzyme in the females, making the difference more obvious. In addition, it is noteworthy that CYP3a activity has also been increased in male in response to DOX but not to a significant level. GH again, may play a role as continuous GH, observed in females, was also found to increase the activity of human CYP3A4, while a decrease in activity has been noticed with pulsatile GH, found in males (Jaffe et al., 2002).

In conclusion, our results have shown that the activity of the enzymes CYP1A2 and CYP3A shows differential extent with sex, and the anticancer drugs DOX and PAX can alter the activity of the metabolizing enzymes of interest. Moreover, these drugs have also shown a significant effect on CYPs in the

previous data (Chapter 4 and 5) at protein level. This emphasizes the importance of performing further studies in such context with more specific substrates, to better understand the levels of alteration and as the alteration in activity can affect drugs response and toxicity.

## Chapter seven: General discussion and future works



## 7 General discussion and future work

### 7.1 General discussion

During drug development stages, the high failure rate of new drug candidates in preclinical or clinical studies due to hepatotoxicity is considered as a substantial problem. Moreover, it became very clear that the unexpected hepatotoxicity is one of the major causes for the withdrawal of drugs from the market. Hence, an urgent need has emerged to develop reliable and robust approaches for predicting the susceptible toxicity and revealing the mechanism by which drug-induced hepatotoxicity develops.

Several *in vivo* and *in vitro* hepatic models have been established and used to investigate the possible mechanisms of drug toxicity on liver. However, the former has provided more sensitive and reliable results, in addition affording insights into the molecular mechanism of drug-induced hepatotoxicity (Suter et al., 2011). In this study, we used the mouse as an animal model that Patterson *et al*, proposed as a highly controllable experimental system, which is extensively used for studying human disease. Moreover, models, in which carefully chosen human cell line xenografts are grown on immune-deficient mice, have been widely used to evaluate the response to a range of anti-cancer drugs (Patterson et al., 2007). Before conducting any experiment on animals, several factors were taken into account in compliance with the 3R's rules including, the dose of drug, duration of exposure, the number of animals, sex, control system, etc. (Parasuraman, 2011).

In order to perform a large-scale analysis of a complex biological system (e.g. liver microsomes) to identify potential biomarkers in response to drugs, an

advanced technique such as quantitative proteomics is necessary. However, identification of low abundance and membranous proteins represent the main challenge for proteomics.

Confirmation of the biomarkers identified by proteomics approach is a crucial step due to possible biological and technical variations (de Gramont et al., 2015). Different approaches such as multiplex reaction monitoring (MRM), Western blotting and ELISA can be used based on the properties of identified biomarkers. Despite the low number of commercially available antibodies and low specificity of others, Western blotting is widely used in the proteomics studies for protein identification and quantification purposes.

Therefore, downstream approach to confirm the change in expression of the particular protein is a key step to building a reliable pipeline in biomarkers development. Different approaches such as multiple reaction monitoring (MRM), Western blotting, enzyme assays and ELISA can be used to support proteomics analysis based on the properties of identified biomarkers. As there are a large number of commercially available antibodies (though frequently of poor specificity), Western blotting is widely used in proteomics studies for protein identification and quantification purposes. Western blotting of MUPs and CYPs, along with VIVID assays specific for selected CYPs has useful provided complementary information to support the proteomics data.

VIVID assay is a high-throughput screening assay that has been designed for examining the inhibitory effect of various compounds on specific baculosomes or bactosomes CYP450 activity. The microtiter plate format that used in VIVID assay, allows analysis of multiple samples simultaneously, providing relatively

high throughput analysis. VIVID assay can monitor the fast conversion of the substrate to product on a fluorescence-based microtiter plate reader, allowing rate change monitoring every minute. Moreover, VIVID assay is relatively quick (30 minutes). However, substrates used in VIVID system are not absolutely specific for each CYP, therefore when analysing heterogeneous mixtures, a number of components could contribute in the assay. In this project, VIVID assay was modified to be used for measuring the activity of selected liver microsomal CYP450s.

Being capable of identification, validation and measuring the activity of hepatic proteins such as CYP450 in this project, provided the opportunity to correlate the quantity of particular proteins with their activities, thus gaining a deeper insight in their response to drug toxicity.

The eventual aim of this thesis was to investigate the effect of selected anti-cancer drugs on liver microsomes through evaluating the alteration in microsomal proteins in response to these drugs and finding out more reliable and sensitive biomarkers of hepatotoxicity. Furthermore, to get a better understanding of the underlying mechanistic basis for chemotherapy-induced liver injury. Therefore, a single dose of the anti-cancer drug was used to treat mouse for 24 hours intraperitoneally, before being killed and organs were collected.

The doses of drugs used in this project were determined in the separated experiment as the maximum tolerated dose by mouse (section 4.2.2.4), which is considered typical in acute toxicological studies. Moreover, it has been concluded that 24 hours of exposure is enough to reduce cell viability in

toxicological studies. Previous transcriptional and proteomics studies showed that 24 hours were enough to induce changes at gene and proteins level, respectively, after DOX administration (Hammer et al., 2010, Wang et al., 2009).

Nevertheless, applying these conditions of treatment was a main challenge in this project. For instance, none of the previous mentioned affected mitochondrial antioxidants by DOX (i.e. CAS, SOD, GSH-Px and TXNDC) was significantly affected in our result. Alshabanah *et al*, have demonstrated significant decreases in CAS, GSH-Px, GSH and SOD activities in rat livers treated with high cumulative dose (18mg/kg/10days) of DOX compared to (10mg/kg/24 hours), whilst, there were no significant changes at low and intermediate cumulative doses (i.e. 6 and 12 mg/kg/10days) (Alshabanah et al., 2010).

Duration of treatment was also considered in TCPOBOP treatment, which has been designed to resemble phenobarbital in CYP induction via CAR activation (Kelley et al., 1985). In this thesis, the effect of TCPOBOP was evaluated and showed comparable results with previous proteomics study (Lane et al., 2007). However, the effect of TCPOBOP was to some extent limited and this might be due to short duration of treatment (3mg/kg/24 hours) compared to Lane *et al*, who used 3mg/kg/4 days (Lane et al., 2007). Moreover, this project highlighted the importance of considering sex variation in further experimental design. A result from 5FU, which has been included in both proteomics studies for reproducibility purpose, showed little overlapping in the number of regulated proteins between both datasets, suggesting the role of sex in different protein responses. Hence, some proteins and their response to drug

or toxicity may be specific to one sex and remain to be clarified. It has been reported in previous studies conducted on rat and mouse livers that the expression of about 1000 genes is sex-dependent (Waxman and Holloway, 2009). For instance, in our results, 9 CYP450 isoforms were uniquely identified in female mice (Set B) and thought to be female-predominant enzymes, while one isoform was uniquely identified in male mice (Set A) (summarized in Figure 4.9). Several studies showed that the expression of human CYP3A4 and its homolog in mouse, which is responsible for metabolizing almost 60% of commercially available drugs, shows sex dimorphism (Diczfalusy et al., 2008, Sakuma et al., 2009, Parkinson et al., 2004). A study by Sakuma *et al*, demonstrated that three isoforms in mouse (CYP3a16, CYP3a41, and CYP3a44), homologous to human CYP3A4, are female-predominant. Consistently CYP3a16 and CYP3a41 were identified in our work and uniquely only in female mice. The authors proposed that the dimorphism in gene expression is not confined to DMEs only, other proteins such as lipoproteins, pheromone binding proteins and fatty acid homeostasis proteins have shown sex dimorphism. Therefore, our study also highlighted MUP, a pheromone binding protein, as a protein of interest. Four isoforms of MUP were identified in male mice compared to 2 identified in female mice, reflecting the naturally occurring higher levels in the former.

As the main limitation of using proteomics is identifying the low abundant and membranous proteins, advances in MS-instrumentation and superior fractionation methodologies were able to enrich and target proteins of interest in the current study, thus overcoming these obstacles. The present project has succeeded in evaluating and optimizing a proteomics workflow that has been

employed in investigating the influence of major anti-cancer drugs on liver microsomes. Moreover, to the best of our knowledge, this research has identified unambiguously the maximum number of CYP450 enzymes including highly homologous isoforms compared to the published literature by using the shotgun approach, making it a valuable approach for studying the influence of drugs on DMEs (particularly CYP450s).

Moreover, MS-based proteomics approach is ideal to study the CYP450 polymorphisms, where particular peptides that contain single nucleotide polymorphism (SNP) can be identified. CYP450s are a well characterised group of enzymes whose activity has been shown to be significantly affected by SNP variants. For example, CYP2D6 which is responsible of metabolism approximately 25% of current drugs showed a large inter-individual variation in the activity, thus variable drug metabolism and drug response (Koski et al., 2007). The CYP2D6 activity ranges significantly within a population and includes ultra-rapid metabolizers (UMs), extensive metabolizers (EMs), intermediate metabolizers (IMs) and poor metabolizers (PMs) (Zhou, 2009). To address this polymorphism, the ProteoGenomics approach uses proteomic data to provide protein-level evidence of gene expression and to help refine gene models. It can help in confirming translated genes, identifying PTMS, and identifying splice variant, mutations, and/or polymorphism. Traditional identification approaches such as Western blotting and activity-based assays cannot define such alterations. However, this project did not correlate the sequence of identified CYP isoforms to their genes. Furthermore, little is known about CYP450 variant in mouse, particularly for cloned mouse. Further

studies concerning ProteoGenomics would be of a high importance applying the advantages of the new MS instruments (Orbitrap Fusion).

The current study was effectively able to detect and quantify bands of CYP1a2 and CYP3a isoforms by using anti-human CYP antibodies. Furthermore, and for the first time, it was able to measure the activity of CYP1a2 and CYP3a from a complex biological sample such as mouse liver microsomes, by the means of VIVID assay.

Traditional proteomics approaches such 2D-DIGE has been used intensively before for evaluating the hepatotoxicity of numerous drugs and validating new biomarkers (Van Summeren et al., 2013, Hammer et al., 2010). However, despite the increasing interest in using the advanced proteomics approaches such as shotgun approach in drug assessment, minor studies were directed to investigate the mechanisms of drug-induced hepatotoxicity

In accordance with previous studies conclusions and our observations, pharmacoproteomics is a powerful technique that can be involved in all aspects of drug development including, drug target discovery. New advancements in MS have increased the sensitivity of detection and resolution (Chapter 1, Table 1.5). However, attempt to identify interesting deregulated proteins in disease or treated tissues is fairly not a straightforward process and a number of obstacles may come across. Therefore, strong challenges in sample preparation, MS-instrumentation, database and statistical analysis need to be considered and handled prior to designing the experiment. One of the important challenges is heating production during proteins extraction for enzyme activity, thus an optimised method was used to overcome it. Another

challenge in pharmacoproteomics and particularly in the present study is the repeatability and reproducibility issue. Such issue is pivotal and considered one of the most critical parts of the biomarker discovery procedure. The costly materials and the long time that iTRAQ and MALDI-TOF MS approaches need were a remarkable obstacle in achieving the reproducibility in the current study.

In conclusion, we propose that pharmacoproteomics is a valuable approach to be used in future toxicological studies and biochemical analysis, aiming to assess the hepatotoxic effect of new candidates as well as already established drugs.



## 7.2 Future work

The data generated from the current study has provided strong evidence about the employment of shotgun proteomics in studying liver microsomes. In addition to giving a preliminary view about the effect of selected drugs on mouse liver, which encourages conducting further verification studies. The main limitations, which were not anticipated at the beginning of the project, were using control mice that were not absolutely mimicking the drug treatments and sex variation between Set A and Set B. However, these limitations were addressed in subsequent studies, where two drugs (DOX and PAX) were selected based on previous results to treat male and female mice with inclusion of solvents and untreated mice. As a comprehensive research conducted for the first time, a number of further experiments would be beneficial, including:

- a. Evaluating the effect of the time course of drug exposure on the regulated proteins, by giving a single dose of an anticancer drug and placebo and scarifying the animal after 24,48 and 96 hours.
- b. Confirming the absolute quantity of identified CYP450 isoforms using selected reaction monitoring (MRM) approach.
- c. Surface plasmon resonance or HPLC approach could be developed to determine if any drugs bind to MUPs, initially using recombinant form.
- d. Employing human cell lines enriched with drug metabolising enzymes, such as HepaRG2 cell line, to correlate the changes in CYPs in our observations with human tissue.

- e. Considering other mouse organs, such as kidney, to determine pharmaco- and toxico-proteomics biomarkers, using analogous proteomics strategies to those used in this project.
- f. Validating other potential biomarkers for their cellular role in drug-induced liver toxicity.
- g. Employing proteomics strategies to analyse the xenograft and the effect of anticancer drugs on it.

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